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LIPID METABOLISM IN ISOLATED RAT SYMPATHETIC NEURONS

by

DEBAO PAN

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A thesis submitted to the Faculty of Graduate Studies and Research in partial fulfillment of the requirements for the degree of MASTER of SCIENCE

in

EXPERIMENTAL MEDICINE

DEPARTMENT OF MEDICINE

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The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research for acceptance, a thesis entitled LIPID METABOLISM IN ISOLATED RAT SYMPATHETIC NEURONS submitted by Debao Pan in partial fulfillment of the requirements for the degree of MASTER OF SCIENCE in EXPERIMENTAL MEDICINE.

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November 27, 1992

ABSTRACT

To investigate whether or not axons were capable of the synthesis of proteins and membrane lipids, a compartmented culture of sympathetic neurons was prepared from the superior cervical ganglia of newborn rats. This model has two advantages compared to the models previously used. First, the neurons were pure and free from other contaminating cell types. Second, three compartments were constructed by the use of a Teflon divider in one dish. The events occurring in axons could be studied independently of those in cell bodies since distal axons could be incubated with medium containing components different from those in the medium used for bathing the cell bodies (plus proximal axons).

The incorporation of radioactivity with [*methyl*-^{3}H]choline, [1-^{3}H]ethanolamine, [3-^{3}H]serine, *myo*-[^{3}H]inositol, [1-^{14}C]acetate into lipids was studied in both cell bodies (plus proximal axons) and axons. The incorporation of radioactivity from each of (i) choline into phosphatidylcholine (PC) and sphingomyelin, (ii) from ethanolamine into phosphatidylethanolamine (PE) and then into PC, (iii) from serine into phosphatidylserine and then into PE, and (iv) from inositol into phosphatidylinositol occured in cell body-containing compartment and in axons. PC and PE are the major lipids in both cell body-containing compartment and axons. Small amounts of all these phospholipids were transported from cell bodies to axons, and vice versa. Although, the incorporation of [^{14}C]acetate into cholesterol, fatty acids and other neutral lipids was observed in cell bodies, no lipids labeled from acetate were found in axons.

The incorporation of L-[^{35}S]methionine into proteins was also examined in both cell bodies and axons by SDS-polyacrylamide gradient gel

electrophoresis. The results showed that only the proteins in cell bodies were labeled by incubation for 16 h with this precursor. No protein synthesis was detected in axons. The labeled proteins were apparently transported from cell bodies to axons by anterograde axonal transport.

Choline kinase, CTP:phosphocholine cytidylyltransferase, and CDP-choline:1,2-diacylglycerol cholinephosphotransferase are the three enzymes involved in PC biosynthesis via the CDP-choline pathway. The results from enzyme assays showed that all three enzymes were present in both cell body-containing compartment and axons. The specific activities of these enzymes were similar to those found in hepatocytes. The specific activity of cytidylyltransferase was the lowest. Pulse-chase studies confirmed that cytidylyltransferase was the rate-limiting enzyme in PC biosynthesis via the CDP-choline pathway in both cell bodies and axons.

Nerve growth factor is an indispensable neurotrophic factor that affects the development and maintenance of sympathetic, sensory and specific populations of neurons in the central nervous system, but its mechanism of action is still unclear. We postulated that nerve growth factor might stimulate the production of PC required for axonal growth. Nerve growth factor stimulated the incorporation of [^{3}H]choline into PC via pulse study. The results from cytidylyltransferase assays, however, suggested that the increase of PC biosynthesis was not caused by an increase of cytidylyltransferase translocation from cytosol to membranes. Therefore, the mechanism for the effect of nerve growth factor on PC biosynthesis needs to be further investigated.

TABLE OF CONTENTS

SIGNED PAGE

ABSTRACT

TABLE OF CONTENTS

LEGEND TO FIGURES

LEGEND TO TABLES

ABBREVIATIONS

ACKNOWLEDGEMENTS

INTRODUCTION 1

1. Neurons and Nerve Growth Factor 1

2. Metabolism of Lipids 8

2.1. Phosphatidylcholine 8

2.1.1. Structure and Biological Role of Phosphatidylcholine 8

2.1.2. PC Biosynthesis 12

2.1.2.1. CDP-Choline Pathway 12

2.1.2.2. Methylation of Phosphatidylethanolamine 17

2.1.2.3. Base Exchange 18

2.1.2.4. Reacylation of Lysophosphatidylcholine 18

2.2. Phosphatidylserine and Phosphatidylethanolamine 20

2.3. Phosphatidylinositol 23

2.4. Cholesterol, Fatty Acids and Other Neutral Lipids 24

3. Thesis Objective 25

MATERIALS AND METHODS 28

1. Chemicals and Radiolabeled Materials 28

2. Culture Materials and the Preparation of Neuron Cultures 28

3. Preparation of Solutions 30

3.1. Buffers for Sample Preparation 30

3.2. Buffers for SDS-Polyacrylamide Gradient Gel Electrophoresis 31

3.3. Solution for Silver-binding Protein Assay 31

3.4. Solutions for CT Assay 31

3.5. Solutions for CPT Assay 32

4. Incorporation of Radiolabeled Precursors into Phospholipids and Their Water-soluble Metabolites in Cell Bodies and Axons 33

4.1. Incorporation of [*methyl*-^{3}H]Choline into PC and SM 33

4.2. Incorporation of [1-^{3}H]Ethanolamine into PE and PC 34

4.3. Incorporation of [3-^{3}H]Serine into PS and PE 34

4.4. Incorporation of *myo*-[^{3}H]Inositol into PI 35

5. Incorporation of [1-^{14}C]Acetate into Cholesterol, Fatty Acids and Other Neutral Lipids 36

6. Incorporation of [^{35}S]Methionine into Proteins 37

7. Assay of Enzymes Involved in PC Biosynthesis via the CDP-Choline Pathway 37

7.1. Preparation of Samples 37

7.2. Protein Assay 38

7.3. Choline Kinase Assay 39

7.4. CTP:Phosphocholine Cytidylyltransferase Assay 39

7.5. CDP-Choline:1,2-Diacylglycerol Cholinephosphotransferase Assay 40

RESULTS 41

1. Establishment of the Cell Culture System 41

2. Incorporation of [*methyl*-^{3}H]Choline into PC and SM in Axons and Cell Bodies of Neurons 43

3. Incorporation of [1-^{3}H]Ethanolamine into PE and PC from PE Methylation in Both Axons and Cell Bodies of Neurons 47

4. The Major Enzymes Involved in PC Biosynthesis via the CDP-Choline Pathway are Present in Both Axons and Cell Bodies 52

5. Incorporation of [3-^{3}H]Serine into PS and PE in Both Axons and Cell Bodies of Neurons 54

6. Incorporation of *myo*-[^{3}H]Inositol into PI in Both Axons and Cell Bodies of Neurons 54

7. Incorporation of [1-^{14}C]Acetate into Cholesterol, Fatty Acids and other Neutral Lipids in Cell Bodies 59

8. Incorporation of [^{35}S]Methionine into Proteins in Cell Bodies of Neurons 62

9. Pulse-chase Studies of Intact Neurons, Cell Bodies and Axons 64

10. Effect of Nerve Growth Factor on the Incorporation of [^{3}H]Choline into PC 66

DISCUSSION 72

1. Biosynthesis of Phospholipids Occurs in Axons of Rat Sympathetic Neurons 72

2. Proteins Can only be Synthesized in Cell Bodies of Pure Sympathetic Neurons 74

3. The Formation of PC from PE Methylation is Significant in Pure Sympathetic Neurons 75

4. PC and PE are the Dominant Phospholipids Synthesized in Pure Sympathetic Neurons 76

5. No Synthesis of Cholesterol or Fatty Acids Could be Detected in Axons of Pure Sympathetic Neurons 77

6. CT is the Rate-limiting Enzyme of PC Biosynthesis in Rat Sympathetic Neurons 78

7. No Direct Effect of NGF on CT Translocation in Neurons 78

REFERENCES 82

LEGEND TO FIGURES

Figure 1. The Schematic Structure of a Secretory Neuron 3

Figure 2. The Structure of Phosphatidylcholine 9

Figure 3. Phosphatidylcholine Cycles and Signal Transduction 11

Figure 4. PC and PE Biosynthetic Pathways and the Regulation of CTP:phosphocholine Cytidylyltransferase Translocation 14

Figure 5. Base Exchange Reaction among Phospholipids 19

Figure 6. The Compartmented Culture Model of Neurons 42

Figure 7. Incorporation of [*methyl*-^{3}H]Choline into PC and SM in Cell Bodies and Axons of Rat Sympathetic Neurons 44

Figure 8. Incorporation of [*methyl*-^{3}H]Choline into Water-soluble Metabolites in Cell Bodies and Axons 46

Figure 9. Incorporation of [1-^{3}H]Ethanolamine into PE and PC of Cell Bodies and Axons 48

Figure 10. Incorporation of [1-^{3}H]Ethanolamine into Water-soluble Metabolites of Cell Bodies and Axons 49

Figure 11. Incorporation of [3-^{3}H]Serine into PS and PE in the Cell Bodies and Axons of Neurons 55

Figure 12. Incorporation of [3-^{3}H]Serine into PS and PE in Axons after the Cell Bodies were Removed from the Center Chamber 56

Figure 13. Incorporation of *myo*-[^{3}H]Inositol into PI in Cell Bodies and Axons of Neurons 57

Figure 14. Incorporation of *myo*-[^{3}H]Inositol into PI in Axons after the Cell Bodies were Removed from the Center Compartment 58

Figure 15. Incorporation of [1-^{14}C]Acetate into Cholesterol and Cholesteryl Esters in Cell Bodies 60

Figure 16. Incorporation of [1-^{14}C]Acetate into Fatty acids, Diacylglycerol and Triacylglycerol in Cell Bodies 61

Figure 17. Incorporation of [^{35}S]Methionine into Neuronal Proteins 63

Figure 18. Pulse-chase Study in Whole Neurons, Cell Bodies and Axons 65

Figure 19. Effect of NGF on PC Biosynthesis in Intact Neurons 67

LEGEND TO TABLES

Table 1. Phospholipid Composition (mole %) of Livers from Several Mammalian Species 21

Table 2. Phospholipid Composition (μmol/g wet wt.) of Developing Rat Brain Tissue 22

Table 3. Amount of Protein of Cell Bodies and Axons from 9-day-old Neurons 50

Table 4. Assay of the Major Enzymes Involved in PC Biosynthesis via the CDP-Choline Pathway in Cell Bodies and Axons of Neurons 53

Table 5. The Effect of NGF on CT Distribution between Cytosol and Membranes in Mass-cultured Neurons 69

Table 6. The Effect of NGF and Anti-NGF Antibody on Distribution of CT Enzyme Activity between Cytosol and Membranes of Mass-cultured Neurons 70

ABBREVIATIONS

ATP	adenosine triphosphate
AX	axons
cAMP	adenosine 3', 5'-monophosphate
CB	cell bodies
CDP-	cytidine diphosphoryl-
CDP-DAG	CDP-diacylglycerol
Ci	Curie
CK	choline kinase
CoA	coenzyme A
CPT	CDP-choline:1,2-diacylglycerol cholinephosphotransferase
CT	CTP:phosphocholine cytidylyltransferase
CTP	cytidine triphosphate
DAG	diacylglycerol
dpm	disintegrations per minute
EDTA	ethylenediamine tetraacetic acid
EGTA	ethyleneglycol-bis-(ß-aminoethyl ether) N', N'-tetraacetate
ER	endoplasmic reticulum
FA	fatty acid
Fig.	Figure
g	gram(s)
g	gravity
G3P	glycerol 3-phosphate
GPC	glycerophosphocholine
h	hour(s)
HMG-CoA	ß-hydroxy-ß-methylglutaryl-CoA
IP	inositol phosphate
IP_2	inositol-4,5-bisphosphate
IP_3	inositol-1,4,5-trisphosphate
kD	kilodaltons
Km	Michaelis-Menten constant
lysoPC	lysophosphatidylcholine
M	molar
mg	milligram
min	minute(s)
ml	milliliter
mM	millimolar
mm	millimeter
mol	mole
myo-I	*myo*-inositol
ng	nanogram
NGF	nerve growth factor
PA	phosphatidic acid
PAP	phosphatidic acid phosphohydrolase
PBS	phosphate-buffered saline
PC 12 cells	pheochromocytoma cells
PC	phosphatidylcholine
PE	phosphatidylethanolamine

PEMT	phosphatidylethanolamine *N*-methyltransferase
PI	phosphatidylinositol
PI-4,5-P_2	phosphatidylinositol-4,5-bisphosphate
PI-4-P	phosphatidylinositol-4-phosphate
PLA_2	phospholipase A_2
PLC	phospholipase C
PLD	phospholipase D
PMSF	phenylmethylsulfonyl fluoride
PS	phosphatidylserine
rpm	revolutions per minute
S	sedimentation coefficient
S.D.	standard deviation
SDS	sodium dodecyl sulfate
SDS-PAGE	SDS-polyacrylamide gradient gel electrophoresis
sec.	second(s)
SM	sphingomyelin
TCA	trichloroacetic acid
TG	triacylglycerol
TLC	thin-layer chromotography
TPA	12-*O*-tetradecanoylphorbol-13-acetate
Tris	tris (hydroxymethyl) aminomethane
v	volume
wt.	weight
µCi	microcurie
µg	microgram
µl	microliter
µm	micrometer
µmol	micromole

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INTRODUCTION

1. NEURONS AND NERVE GROWTH FACTOR

The vertebrate nervous system includes the central nervous system and the peripheral nervous system. The central nervous system comprises the brain and spinal cord, and the peripheral nervous system is composed of the cranial and spinal nervous systems associated with the brain and spinal cord, respectively (Littell, 1990).

Nerve cells (neurons) can receive, conduct and transmit signals. In the central nervous systems, most of the nerve cells are interneurons which form a complex network to combine and process sensory information as well as to control motor output. In the peripheral nervous system, three types of neurons can be functionally distinguished: the sensory neurons carry the information inward to the central nervous system, the autonomic neurons carry the commands outward to their effectors, and the motor neurons carry the commands to the skeletal muscles. In the motor neurons the nerve cell bodies lie inside the central nervous system that controls skeletal muscles, whereas in the sensory neurons and autonomic neurons the cell bodies lie outside of central nervous system (Littell, 1990).

The peripheral autonomic system is further subdivided into sympathetic and parasympathetic sections. The preganglionic axons of sympathetic neurons emerge from the thoracic and lumbar segments of the spinal cord, terminate in sympathetic ganglia, and innvervate postganglionic neurons. Postganglionic axons innvervate effectors such as glands (sweat, salivary, and digestive), the smooth muscles in all organs (blood vesssels, viscera, excretory

organs, genitalia, lungs, hair, and eyes), some cells (adipose, liver cells) and heart (Atwood and Mackay, 1989). The parasympathetic axons emerge from the brainstem and sacral segments of the spinal cord, go directly to effector organs, and innervate neurons within or close to the organs. These parasympathetic postganglionic neurons then innervate the target organs, such as smooth muscles of gastrointestinal organs, excretory organs, genitalia, lungs, glands of these organs, atria of the heart, tear and salivary glands, and intraocular muscles (Atwood and Mackay, 1989).

Neurons have highly asymmetrical morphology in terms of both the volume and dimensions as well as the obviously regional specialization (Alberts et al., 1989). Typically, three major portions of a neuron can be distinguished: the cell body, the dendrites, and the axon (Fig. 1). The length of the specialized extensions (dendrites, axons and nerve endings) may exceed that of the cell body by several orders of magnitude, especially in the peripheral nervous system. For example, a large sciatic motor neuron in a human being may have an axon 15 μm in diameter and a meter in length (Alberts et al., 1989). The cell body of a neuron contains a nucleus, as well as ribosomes, endoplasmic reticulum, mitochondria, and Golgi apparatus. Although dendrites contain some ribosomes, no ribosomes have been observed in the axon (Steward and Levy, 1982; Davis et al., 1987). The dendrites are a set of branching, tubular processes that extend like antennae from the cell body and provide an enlarged surface area for the reception of signals from other cells. The axon is a cell process which is generally single and longer than the dendrites and conducts action potentials away from the cell body to distant targets. The axon commonly divides at its far end into many branches, distributing its signals to many destinations simultaneously. The internal

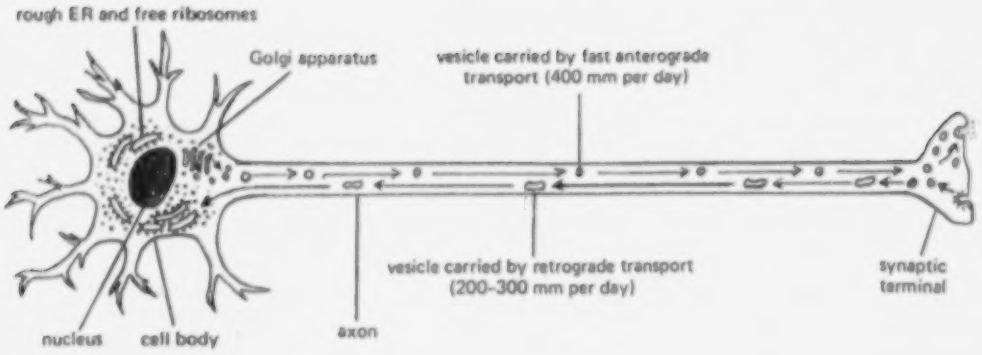

rough ER and free ribosomes
Golgi apparatus
vesicle carried by fast anterograde transport (400 mm per day)
vesicle carried by retrograde transport (200-300 mm per day)
synaptic terminal
nucleus
cell body
axon

Figure 1. The Schematic Structure of A Secretory Neuron

This neuron is viewed schematically as a secretory cell in which the axon terminal lies at a great distance from the cell body. This mode of organization creates a need for a rapid axonal transport mechanism since most macromolecules are synthesized in the cell body. The synaptic vesicles formed in cell body have to be transported from the cell body to axon, but in most neurons synaptic vesicles are believed to be formed largely by local recycling of membrane in the axonal terminal. (Modified from B. Alberts, D. Bray, J. Lewis, M. Raff, K. Roberts, and J.D. Watson, *Molecular Biology of The Cell*, 2nd edition, 1989 pp.1064)

strength of the long thin processes (dendrites and axons) is provided by the neurofilaments and microtubules in the cytoplasm of all nerve cell processes (Cleveland and Hoffman, 1991).

It had long been believed that only the cell body is an active biosynthetic center since most of the ribosomes, rough endoplasmic reticulum and Golgi apparatus in a neuron are confined to the cell body (Steward and Levy, 1982; Alberts et al., 1989). Therefore, all materials (e.g. proteins and lipids) needed for axonal growth, membrane maintenance and regeneration must be synthesized in the cell body and then delivered to the axon by axonal transport (Alberts et al., 1989; Morin et al., 1991). Three types of axonal transport have been identified in neurons: slow anterograde axonal transport, fast anterograde transport, and retrograde axonal transport (Okabe and Hirokawa, 1989). Cytoskeletal proteins and cytosolic proteins, including many enzymes, are delivered by slow axonal transport at speeds of 1 to 5 mm/day. Noncytosolic materials, such as mitochondria, and the secreted and membrane-associated proteins, are delivered by fast anterograde transport at speeds of 200-400 mm/day. Retrograde axonal transport carries aging mitochondria, lysosomal hydrolases, nerve growth factor, and other materials obtained by endocytosis from axonal terminals back to cell body at the speed of about 200-300 mm/day (Fig. 1). Sometimes both fast anterograde, and fast retrograde, transport are together named as fast axonal transport (Cooper and Smith, 1974).

Nerve growth factor (NGF) is the most important neurotrophic factor we know so far. The first evidence suggesting the existence of NGF came from a transplant experiment in which mouse sarcoma 180 (S_{180}) cells were transplanted into a chick embryo (Buker, 1948). In this experiment, the

growing tumor in the embryo was quickly invaded by nerve bundles. The ipsilateral dorsal root ganglia at the level of the tumor were also enlarged compared to the contralateral ganglia. A further experiment showed that sensory ganglia, as well as sympathetic ganglia in the vicinity of the implanted sarcoma were obviously enlarged compared to more distant ganglia (Levi-Montalcini and Hamburger, 1951; Levi-Montalcini and Hamburger, 1953). Because in these circumstances the only link between the tumor and the nervous system was the circulation, the effect had to be mediated by a substance secreted into the blood by the tumor. Therefore, this hypothetical substance was named nerve growth factor (Levi-Montalcini and Hamburger, 1951; Levi-Montalcini and Hamburger, 1953). The existence of this factor in the tumor tissues was later demonstrated by Levi-Montalcini (Levi-Montalcini et al., 1954)

NGF is also found in innervated mammalian tissues (Korsching and Thoenen, 1983), snake venom (Cohen and Levi-Montalcini, 1956), and male mice salivary glands (Cohen, 1960). The discovery of a large amount of NGF in male mice salivary glands is not just a fascinating story but also gave researchers a good chance to isolate and characterize this molecule (Bocchini and Angeletti, 1969).

NGF isolated from the male mouse salivary glands is a complex of α, β, and γ subunits in a ratio of $2\alpha:\beta:2\gamma$ (Bocchini and Angeletti, 1969). The molecular weight of the complex is about 130 kD and the sedimentation coefficient is 7 S (Varon et al., 1968). The α subunit is 26 kD. The γ subunit has about same molecular weight as the α subunit (Varon et al., 1968; Bocchini and Angeletti, 1969). Neither the α nor the γ moiety has obvious effects on neurons and both of their physiological roles are still obscure. Only the β subunit

promotes neurite outgrowth and cell survival (Greene et al., 1971). The β subunit is also known as 2.5 S NGF which consists of two identical monomers, each of which comprises 118 amino acids; these form a noncovalently-bound dimer with a molecular weight of 26,518 daltons (Angeletti and Bradshaw, 1971). Evidence concerning the distribution and concentration of NGF is difficult to obtain because very low quantities of NGF exist in most tissues. Using a two-site enzyme immunoassay, researchers have demonstrated that NGF is indeed present in the targets of sympathetic neurons (Korsching and Thoenen, 1983). The levels of NGF measured are in the range of 1 ng/g of target tissue. Tissues not well innervated by sympathetic fibers, such as heart ventricle and skeletal muscle, have amounts of NGF below the detection limit level (about 0.3 ng/g).

The mechanism of action of NGF on neurons is still not clear although NGF was discovered four decades ago (Kaplan et al., 1991a). We do know that the sympathetic neurons, sensory neurons and some selective populations of cholinergic neurons in the central nervous system require NGF for their differentiation and survival, whereas other types of neurons do not (Tischler and Greene, 1975; Kaplan et al., 1991a). NGF also has important effects on some non-neural cells. For example, when pheochromocytoma cells (PC12 cells) are treated with NGF, the cells send out neurites and show catecholamine-specific fluorescence (Greene and Tischler, 1976). NGF also increases the number of mast cells in neonatal rats but the mechanism is not clear (Aloe and Levi-Montalcini, 1977).

NGF stimulates the synthesis of RNA and some proteins, affects ion fluxes, and affects the uptake of small molecules, such as catecholamines (Greene and Tischler, 1976; Greene and Shooter, 1980; Yanker and Shooter,

1982). Specific NGF receptors have been found on the membranes of NGF-sensitive nerve endings and these receptors are generally absent from NGF-unresponsive nerve endings (Greene and Shooter, 1980). NGF can bind with its receptors, and then is taken up and retrogradely transported to the cell body (Purves and Lichtman, 1985). For example, when ^{125}I-NGF is injected into a rat eye, the radioactive NGF interacts with NGF receptors on the nerve endings, then is internalized and transported to the cell body where it arrives in its native form (Bradshaw, 1978; Greene and Shooter, 1980; Schwab and Thoenen, 1983). After transport into the cell body NGF does not go into the nucleus but remains in the cytoplasmic compartment (Greene and Shooter, 1980; Yanker and Shooter, 1982). NGF does not produce its effects by subsequent release from cytoplasmic compartments because direct intracellular introduction of NGF fails to produce neurite outgrowth from pheochromocytoma cells that normally respond to external NGF (Heumann et al., 1981; Seeley et al., 1983).

There is other evidence showing that NGF has local control of neurites (Campenot, 1977). When neurons are grown in the central compartment of a three-chamber culture dish (see Fig. 6 and Materials and Methods for details), the neurites grow under the barrier dividing the compartments and into the lateral chambers only when all chambers contain an adequate concentration of NGF (about 10 ng/ml). Removal of NGF from one or both of the side chambers causes a local regression of neurites without affecting the survival of the cells in the center compartment (Campenot, 1977). These results suggest that some kind of local transduction mechanism may exist in axon endings to transmit neurotrophic signals (Campenot, 1977; Campenot et al., 1991).

Interaction of NGF with a cell receptor is required for the transmission of neurotrophic signals within the cells, but the mechanisms by which NGF

elicits its neurotrophic effects in the cells have not been fully resolved (Kaplan et al., 1991b). Two NGF receptors have been found so far. A 75 kD receptor ($p75^{NGF}$) that binds NGF has been identified and cloned and is present in NGF-responsive cells (Chao et al., 1986; Radeke et al., 1987). This is a low affinity receptor and appears not to be involved in signal transduction. (Chao et al., 1986; Radeke et al., 1987). The other NGF receptor is a 140 kD protein ($p140^{prototrk}$). This receptor is a membrane-spanning tyrosine kinase (*trk* protein) and is expressed only in neural tissues (Kaplan et al., 1991b). The phosphorylation of *trk* protein on tyrosine residue(s) in response to NGF binding is a specific and fast reaction that can occur with physiological quantities (picomolar) of NGF (Kaplan et al., 1991a; Kaplan et al., 1991b). Athough, NGF may cause its effects by binding to this receptor on sensory neurons as a possible mechanism, the 140 kD *trk* protein has not been found in sympathetic neurons (Kaplan et al., 1991a). Therefore, the mechanism of action of NGF still needs to be further investigated.

2. METABOLISM OF LIPIDS

2.1. Phosphatidylcholine

2.1.1. Structure and Biological Role of Phosphatidylcholine

Phosphatidylcholine (PC) is quantitatively the most important phospholipid in all eukaryotic cells, including cells of the mammalian nervous system (Sun and Foudin, 1985). The structure of PC consists of two fatty acids in ester linkages at the *sn*-1 and *sn*-2 positions of glycerol, which give rise to two hydrophobic hydrocarbon tails, and a choline phosphate residue attached to the *sn*-3 position, which is the hydrophilic head group. The chain length and

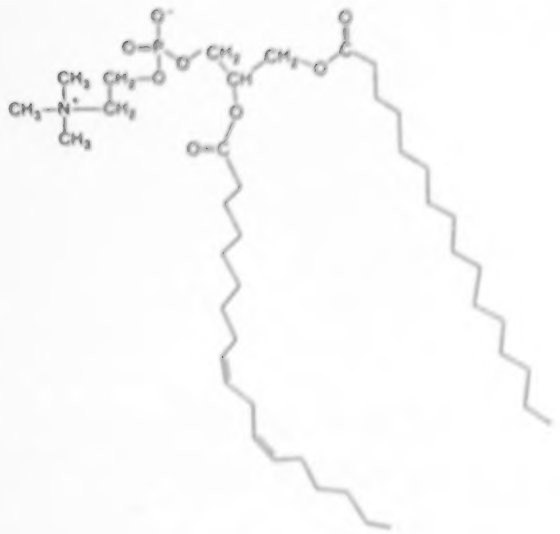


Figure 2. The Structure of Phosphatidylcholine

Phosphatidylcholine consists of two fatty acids in ester linkages at the *sn*-1 and *sn*-2 positions of glycerol, which give rise to two hydrophobic hydrocarbon tails, and a choline phosphate residue attached to the *sn*-3 position, which gives rise to a hydrophilic head group. The chain length and the degree of unsaturation of fatty acids in phosphatidylcholine vary in molecular species, but the fatty acid at the *sn*-1 position is usually saturated whereas that at the *sn*-2 position is usually unsaturated.

the degree of unsaturation of fatty acids in PC vary in molecular species, but the fatty acid at the *sn*-1 position is usually saturated whereas that at the *sn*-2 position is usually unsaturated (Fig. 2).

PC is crucial for eukaryotic cell survival (Esko et al., 1981; Esko et al., 1982). In mammals, a major biological role of PC is as the main structural component of cell membranes. This is because PC, like the other amphipathic phospholipids, tends to arrange spontaneously into a bilayer when it is suspended in an aqueous environment (Cullis and Hope, 1991). The lipid bilayer in cells serves both as a permeability barrier and as a matrix for embedded membrane proteins, therefore providing an appropriate environment for the maintenance of functional protein conformation (Cullis and Hope, 1991). Although most membrane functions are mediated by proteins, PC and other phospholipids have been implicated in cellular events such as membrane fusion (De Kruiff et al., 1980), endocytosis (Schroeder, 1981) and generation of second messengers (Pelech and Vance, 1989; Exton, 1990).

In animals, PC is a constituent of bile, plasma lipoproteins and lung surfactant as well as cellular membranes. Biliary PC assists in the solubilization of triacylglycerols and therefore facilitates the digestion of triacylglycerols in the small intestine (Brindley, 1991). PC and other phospholipids in the plasma lipoproteins are present in a surface monolayer thus rendering the core of triacylglycerols and cholesterol esters water-soluble (Davis, 1991). Even the secretion of very low density lipoproteins requires active PC synthesis in the liver (Yao and Vance, 1988). Phospholipids are the major components of lung surfactant in which approximately 80% of phospholipids is PC by weight (King, 1984; Possmayer, 1984). Specifically, about half of surfactant PC is present as the *sn*-1,2-dipalmitoyl PC which lines

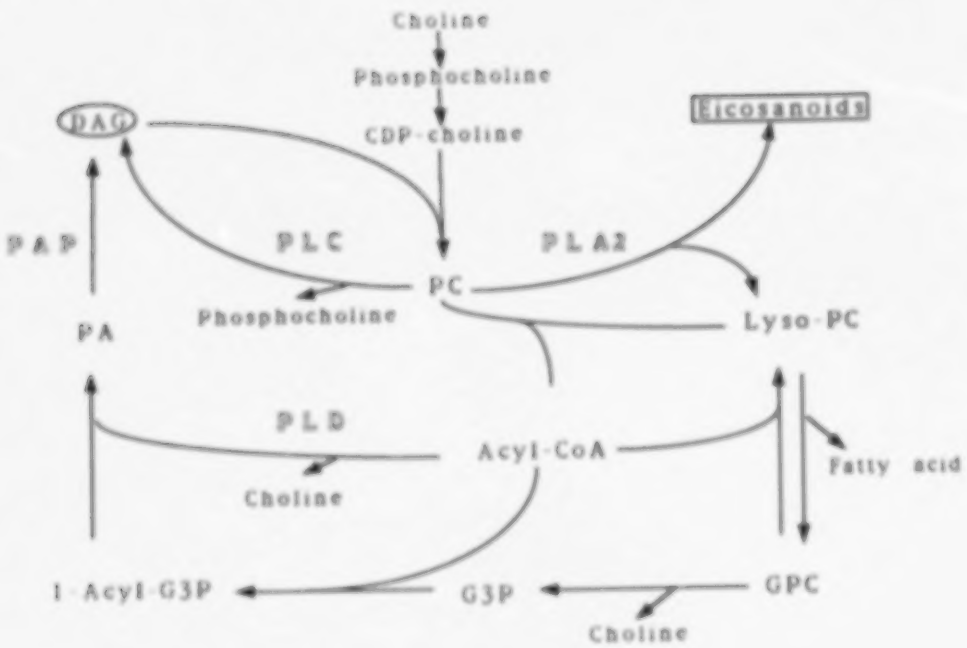


Figure 3. Phosphatidylcholine Cycles and Signal Transduction

Phosphatidylcholine cycles for generation of second messengers diacylglycerol and eicosanoids. Diacylglycerol (DAG) can be generated directly via phospholipase C (PLC) or by the action of phospholipase D (PLD) to yield phosphatidic acid (PA), which is cleaved to DAG by PA phosphohydrolase (PAP). Alternatively, the phospholipase A_2 (PLA_2)-catalysed hydrolysis of PC provides for the production of lyso-PC which may be re-esterified to PC or catabolized to glycerophosphocholine (GPC), which can be further degraded to glycerol 3-phosphate (G3P) and choline. G3P can be converted back to DAG via PA synthesis. DAG can react with CDP-choline to complete PC turnover cycle. [Modified from Vance, D.E. (1991) *Biochemistry of Lipids, Lipoproteins and Membranes* Eds. D.E. Vance and J. Vance, Elsevier, pp. 232]

the surface of alveoli to lower surface tension and prevent alveolar collapse during expiration (King, 1984; Possmayer, 1984).

Moreover, PC metabolism is also involved in signal transduction. Certain hormones, growth factors, and phorbol esters stimulate PC biosynthesis (Mufson et al., 1981; Pelech and Vance, 1984). Apparently, guanine nucleotide-binding proteins are coupled with agonist receptors and consequently phospholipases A_2, C and D are activated, which can initiate a PC cycle (Fig. 3). In this cycle, PC catabolism generates diacylglycerol which is one of the most important second messengers, and arachidonic acid which is an immediate precursor of eicosanoids. Diacylglycerol can be generated from PC directly via phospholipase C or indirectly by the combined actions of phospholipase D and phosphatidate phosphohydrolase (Pelech and Vance, 1989).

2.1.2. PC Biosynthesis

In mammals, all cells except erythrocytes are able to synthesize PC from fundamental components, i.e., glycerol, phosphate, choline and fatty acids. There are four pathways for the synthesis of PC: (i) the CDP-choline pathway (Kennedy pathway); (ii) the stepwise methylation of phosphatidylethanolamine; (iii) base exchange; (iv) the acylation of lysophosphatidylcholine.

2.1.2.1. CDP-Choline Pathway

This pathway is also called Kennedy pathway after Eugene P. Kennedy who discovered it in the 1950s (Kennedy, 1962). This is the major route to synthesize PC in liver (Sundler and Åkesson, 1975a; Sundler and Åkesson,

1975b) and brain tissues (Ansell and Spanner, 1968). About 60 to 80% of PC in rat liver is synthesized via this pathway (Sundler and Åkesson, 1975a; Sundler and Åkesson, 1975b). The synthesis of PC by this pathway involves the sequential conversion of choline to phosphocholine, to CDP-choline and then to PC (Fig. 4).

The first enzyme in this pathway is choline kinase (EC 2.7.1.32) which is a cytosolic, Mg^{++}-ATP-dependent enzyme. It has been purified to homogeneity from rat kidney (Ishidate et al., 1984) and liver (Porter and Kent, 1990). The enzyme from kidney appears to exist as a dimer of two 42 kD subunits. The pH optimum is 8.5-9.0 with an apparent Km of 110 μM for choline and 1.5 mM for Mg^{++}-ATP (Ishidate, 1989). Three isoenzymes recovered from rat liver have similar properties (Ishidate, 1989). It has been confirmed that the phosphorylation of both choline and ethanolamine are catalyzed by the same enzyme in rat kidney, lung, liver, and intestine as well as in yeast (Ishidate et al., 1985). The structural gene for choline kinase has also been cloned from yeast (Hosaka et al., 1989) and the cDNA from rat liver (Uchida and Yamashita, 1992).

The second enzyme in the CDP-choline pathway is CTP:phosphocholine cytidylyltransferase (EC 2.7.7.15) which is a Mg^{++}-dependent enzyme that catalyzes the synthesis of CDP-choline and pyrophosphate from CTP and phosphocholine. Under most metabolic conditions cytidylyltransferase (CT) acts as the rate-limiting and regulated enzyme of PC biosynthesis by the CDP-choline pathway (Pelech and Vance, 1989; Vance, D.E., 1989b).

CT has been purified (Feldman and Weinhold, 1987) and cloned (Kalmar et al., 1990) from rat liver. The purified CT from rat liver has a molecular weight of 41,720 daltons and in cytosol it appears to exist as a dimer of this

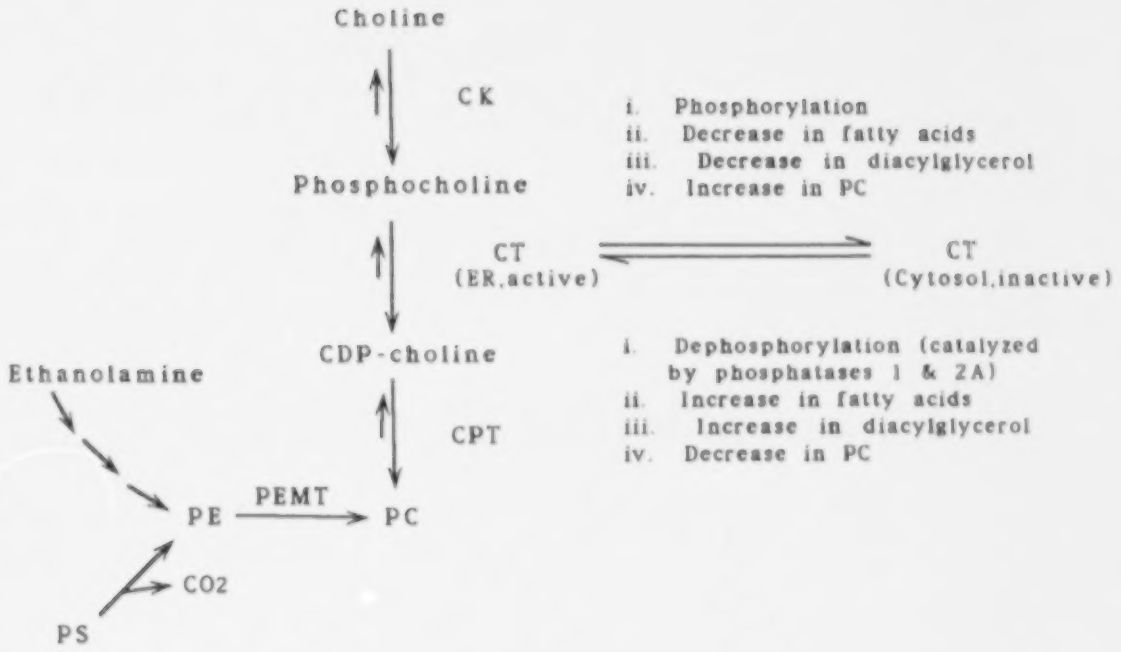


Figure 4. PC and PE Biosynthetic Pathways and The Regulation of CTP:phosphocholine Cytidylyltransferase Translocation

The major PC biosynthetic pathway in liver, nervous system and most other tissues is the CDP-choline pathway in which choline is sequentially converted to phosphocholine, to CDP-choline, and then to PC. An alternative PC biosynthetic pathway is the methylation of PE in which three methyl groups are added to PE to give rise to PC. PE is mainly synthesized from ethanolamine via the CDP-ethanolamine pathway or from decarboxylation of phosphatidylserine (PS). In the CDP-choline pathway, the enzyme CTP:phosphocholine cytidylyltransferase (CT) acts as the rate-limiting and regulated enzyme of the PC biosynthetic process. CT exists both in the cytosol as an inactive reservoir of enzyme and on the membranes as an active form of the enzyme. CT activity is regulated by reversible translocation of the enzyme between the cytosol and the membranes. The major factors governing CT translocation are shown. PEMT, *phosphatidylethanolamine N-methyltransferase*; CK, *choline kinase*; CPT, *CDP-choline:1,2-diacylglycerol cholinephosphotransferase*.

subunit (Weinhold et al., 1986; Feldman and Weinhold, 1987). The pH optimum for CT is between 6.0-7.0 (Pelech and Vance, 1984). CT exists both in the cytosol as an inactive reservoir of enzyme, and on endoplasmic reticulum as the active form of the enzyme (Sleight and Kent, 1980; Vance, D.E., 1989a). Recent immunofluorescence (Watkins and Kent, 1992) and subcellular fractionation (Morand and Kent, 1989) studies reported that the enzyme was primarily associated with the nuclear membrane of Chinese hamster ovary cells although this needs to be confirmed. The soluble enzyme can be activated *in vitro* by some phospholipids such as lysophosphatidylethanolamine, phosphatidylserine, phosphatidylglycerol, and phosphatidylinositol (Pelech and Vance, 1984; Choy, 1978).

CT activity *in vivo* is regulated by a reversible translocation between the cytosol, where the enzyme is inactive, and the membranes, where the enzyme is activated (Fig. 4). Four major factors governing CT distribution and activity have been studied:

First, the phosphorylation/dephosphorylation of CT might be a possible mechanism in controlling CT translocation therefore governing PC biosynthesis. An increase in the phosphorylation state of CT in hepatocytes is associated with an attenuation of PC biosynthesis (Vance, D.E., 1990; Kent, 1991). Okadaic acid, a complex polyketal, is a potent and specific inhibitor of phosphoprotein phosphatases 1 and 2A (Bialojan and Takai, 1988). Okadaic acid decrease PC biosynthesis and this coincides directly with an increased phosphorylation of cytosolic CT (Hatch, et al., 1992). Addition of partially purified catalytic subunits of phosphoprotein phosphatases 1 and/or 2A promoted the association of purified CT with washed microsomal membranes in a concentration dependent manner (Hatch, et al., 1991).

Second, fatty acids promote the binding of CT to membranes (Pelech et al., 1983). In 1 mM oleate-treated rat hepatocytes, the microsomal CT activity was doubled with a corresponding reduction of the cytosolic enzyme activity. Removal of the fatty acids dissociates the enzyme from the membranes (Pelech et al., 1983; Cornell and Vance, 1987).

Third, diacylglycerol may be an important mediator of CT translocation. Translocation of CT from cytosol to membranes is inhibited by cAMP analogues which decrease the level of diacylglycerol (Jamil et al., 1992). On the other hand, 12-*O*-tetradecanoylphorbol-13-acetate (TPA) and related phorbol esters stimulate CT translocation from cytosol to membranes and therefore stimulate PC biosynthesis in HeLa cells (Pelech, et al., 1984; Watkins and Kent, 1990). Although phorbol esters are the activators of protein kinase C, new evidence has demonstrated that phorbol esters stimulate the level of cellular diacylglycerol which causes the activation of CT. Protein kinase C is not involved in this process (Utal et al., 1991).

Fourth, the concentration of PC in cellular membranes feedback regulates the distribution of CT between cytosol and membranes. A decrease in PC on the membranes causes the enzyme to be activated by binding to the membrane, whereas an increase in PC on the membranes causes the release of enzyme into the cytosol (Vance, D.E., 1989a; Vance, D.E., 1990).

The third enzyme in the CDP-choline pathway is CDP-choline:1,2-diacylglycerol cholinephosphotransferase (EC 2.7.8.2). This enzyme exists mostly on the cytosolic side of the endoplasmic reticulum of rat liver (Vance, D.E. et al., 1977; Coleman and Bell, 1978) and Golgi (Vance, J.E. and Vance, D.E., 1988). It was also found in the nuclear membranes of neuronal nuclei isolated from immature rabbit cerebral cortex (Baker and Chang, 1982). The rate of this

enzyme reaction *in vivo* is regulated by the supply of CDP-choline (Vance, D.E., 1989b) and diacylglycerol (Jamil et al., 1992). Cholinephosphotransferase has been cloned from yeast (Hjelmstad & Bell, 1990) but only partial purification of this enzyme from liver has been achieved so far (O and Choy, 1990). Cholinephosphotransferase from yeast has 407 amino acids with a predicted molecular weight of 46,305 daltons (Hjelmstad and Bell, 1990).

2.1.2.2. Methylation of Phosphatidylethanolamine

The phosphatidylethanolamine (PE) methylation pathway is an alternative PC biosynthesis pathway which is quantitatively significant only in liver (Ridgway and Vance, 1987) but not in other tissues (Ridgway, 1989). In liver, about 20 - 40% PC is synthesized by this pathway (Sundler and Åkesson, 1975a; Sundler and Åkesson, 1975b). The formation of PC is achieved by the sequential transfer of three methyl groups from *S*-adenosylmethionine to PE (Fig. 4). All three methylation reactions in rat liver are catalyzed by phosphatidylethanolamine *N*-methyltransferase (PEMT) (Ridgway, 1989), a membrane-bound enzyme of the ER and Golgi (Vance, J.E. and Vance, D.E, 1988). Two cDNAs for PEMT have been cloned from rat liver with calculated molecular weights 21 kD and 23 kD (Cui and Vance, unpublished results). In yeast there are two phosphatidyethanolamine methyltransferases involved in the methylation of PE to PC (Kodaki and Yamashita, 1987). The first gene, PEM 1, codes for an enzyme with a calculated molecular weight of 101,202 that catalyzes only the methylation of PE to phosphatidyl-*N*-monomethylethanolamine (Kodaki and Yamashita, 1987). The second gene, PEM 2, codes for an enzyme with a calculated molecular weight of 23,150

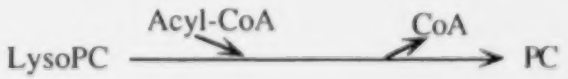
daltons that is capable of the conversion of PE to PC (Kodaki and Yamashita, 1987).

2.1.2.3. Base Exchange

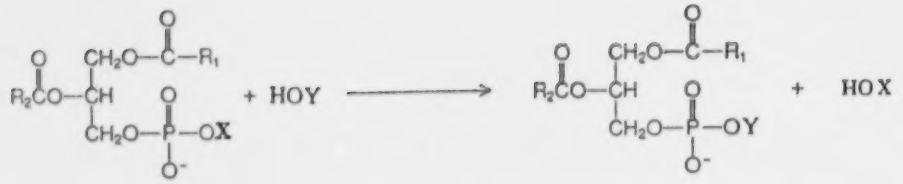
In a third pathway of PC biosynthesis, choline exchanges with the headgroup of a preexisting phospholipid to yield PC (Kanfer, 1989) (Fig. 5). This pathway plays a quantitatively minor role in PC biosynthesis and appears to be active primarily in liver and brain tissues (Porcellati et al., 1971; Kanfer, 1980; Filler and Weinhold, 1980). The base-exchange reaction requires calcium and is an energy-independent reaction (Kanfer, 1980). The choline exchange activity in intact rat brain microsomes is trypsin-sensitive and is therefore located on the cytosolic side of these vesicles (Buchanan and Kanfer, 1980).

2.1.2.4. Reacylation of Lysophosphatidylcholine

In mammalian tissues, lysophosphatidylcholine (lysoPC) may be acylated to PC by two types of reactions (Choy and Arthur, 1989). The first is the transfer of an acyl group from acyl-CoA to lysoPC and is catalyzed by lysoPC:acyl-CoA acyltransferase.



This acylation pathway exists in cytosolic, microsomal, mitochondrial, and nuclear fractions of a variety of tissues (Baker and Chang, 1981; Needleman et al., 1985; Arthur and Choy, 1986). The second pathway is a transacylation reaction in which an acyl group is directly transferred from a



Acceptor Phospholipid

Phosphatidyl-Y

Figure 5. Base Exchange Reaction among Phospholipids

Phospholipids can be synthesized via a base exchange reaction among phospholipids themselves. In this reaction **X** and **Y** may be choline, serine, ethanolamine, monomethylethanolamine, or dimethylethanolamine.

donor phospholipid (including lysoPC) to another lysoPC molecule without the release of fatty acid by phospholipase (Choy and Arthur, 1989).

It has been suggested that the high concentrations of polyunsaturated fatty acids found in certain species of phospholipids are achieved via transacylation (Choy and Arthur, 1989). Also the synthesis of PC by lysoPC-lysoPC transacylation may play a significant role in the biosynthesis of 1,2-dipalmitoyl-PC in the lungs (Batenburg et al., 1979; De Vries et al., 1985).

2.2. Phosphatidylserine and Phosphatidylethanolamine

Phosphatidylserine (PS) and PE are other important phospholipids in mammalian cells (Cullis and Hope, 1991). In rat liver, PS and PE account for about 3% and 25% of the total phospholipids, respectively (Table 1) (Padley et al., 1986). The content of these two lipids is much higher in adult rat brain (Table 2) (Sun and Foudin, 1985).

The PS synthesis pathway in *E. coli* is different from that in mammalian cells. PS can be synthesized in *E. coli* directly from serine and CDP-diacylglycerol (Zubay, 1988). However, in mammalian cells such as Chinese hamster ovary cells PS synthesis occurs via the base-exchange reaction (Fig. 5). The process is catalyzed by two serine-exchange enzymes I and II, (Kuge et al., 1985). The base-exchange enzymes have been found in rat liver and brain (Pullarkat et al., 1981; Taki, 1978; Baranska, 1988). The storage phospholipid substrate for PS synthesis can be PC, PE or other phospholipids (Kuge et al., 1986a; Kuge et al., 1986b). PS is subsequently decarboxylated to PE on the outer aspect of the inner mitochondrial membranes by PS decarboxylase (Voelker, 1989a; Voelker, 1989b). In some mammalian cells a significant proportion of PE

Table 1 Phospholipid Composition (mole %) of Livers from Several Mammalian Species (Modified from F.B. Padley, F.D. Gunstone and J.L. Harwood, The Lipid Handbook, Eds. F.D. Gunstone, J.L. Harwood and F.B. Padley, 1986, pp. 49-170)

Phospholipid	Human	Rat	Sheep	Bovine	Mouse
Phosphatidylcholine*	43.6	50.8	40.5	55.7	45.6
Phosphatidylethanolamine*	27.9	25.2	31.5	13.0	25.1
Phosphatidylinositol	8.6	7.2	8.2	7.9	7.7
Phosphatidylserine	3.1	3.2	2.3	4.2	3.9
Phosphatidylglycerol	nd**	nd	5.3	nd	0.1
Diphosphatidylglycerol	3.7	4.8	1.1	4.1	4.7
Sphingomyelin	4.6	4.2	5.0	5.8	4.7
Others	8.5	5.6	6.1	9.3	8.2

* Includes plasmalogen derivatives

** Not determined

Table 2. Phospholipid Composition (μmol/g wet wt.) of Developing Rat Brain Tissue

Phospholipid	3*	12	24	42	180
Phosphatidylcholine	14.7	20.4	24.8	25.0	25.0
Phosphatidylethanolamine	5.3	8.0	9.4	10.9	10.7
Ethanolamine plasmalogen	2.2	4.7	11.3	13.5	13.0
Phosphatidylinositol	1.2	1.6	2.0	2.2	2.2
Phosphatidylserine	2.9	4.4	7.0	8.3	8.5
Phosphatidic acid	0.1	0.3	0.7	1.0	1.3
Sphingomyelin	0.2	1.0	3.2	3.6	3.7

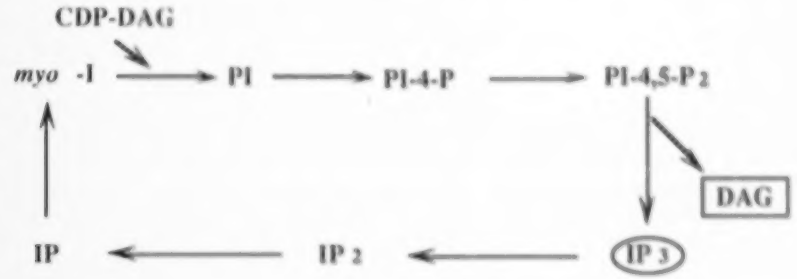
* Age (days) after birth

is synthesized via the action of PS decarboxylase (Voelker, 1989a; Voelker, 1989b; Kuge et al., 1986a; Kuge et al., 1986b). Therefore, a cycle of net convertion of serine to ethanolamine occurs. This appears to be a major route for the biosynthesis of ethanolamine in eukaryotic cells (Vance, J.E., 1991).

In addition to the decarboxylation of PS, PE can also be synthesized by two other pathways, i.e. the CDP-ethanolamine pathway (Fig. 4) and the calcium-stimulated base-exchange pathway (Fig. 5). In the CDP-ethanolamine pathway, PE is synthesized from ethanolamine via phosphoethanolamine and CDP-ethanolamine (Fig. 4) (Kennedy and Weiss, 1956). In the base-exchange pathway, PE is synthesized by the exchange of ethanolamine with the base-moiety of pre-existing phospholipids (Fig. 5) (Sundler et al., 1974).

2.3. Phosphatidylinositol

Phosphatidylinositol (PI) accounts for about 5% of the lipids present in animal cell membranes (Zubay, 1988). A large number of structurally related PI molecules, including over 63 distinct inositol phosphate species, has been found (Downes, 1989). Some of them, at much lower concentrations (1-3% of PI), are phosphatidylinositol-4-phosphate (PI-4-P) and phosphatidylinositol-4,5-bisphosphate (PI-4,5-P_2) (Zubay, 1988).



PI is synthesized from *myo*-inositol (*myo*-I) and CDP-diacylglycerol (CDP-DAG) (Downes and Macphee, 1990). The most important aspect of PI metabolism is that some PIs are involved in signal transduction. Extracellular signals by activators, such as neurotransmitters and peptide hormones, flow from the cell surface into the cells and these signals are bridged by inositol-containing phospholipid turnover (Downes and Macphee, 1990). A "PI cycle" is created in which the phosphatidylinositol-4,5-trisphosphate (PI-4,5-P_3) on the plasma membrane is degraded to inositol-1,4,5-trisphosphate (IP_3) and diacylglycerol (DAG) by a PI-specific phospholipase C. Both of the degradation products of PI-4,5-P_3 have important regulatory functions (Heacock et al.,1990). Diacylglycerol activates protein kinase C which then phosphorylates serine and threonine residues in many target proteins. IP_3 is involved in mobilization of calcium from intracellular stores (ER) and the rise in cytosolic calcium can activate certain enzymes. For example, protein kinase C is enzymatically active only in the presence of Ca^{2+} and phosphatidylserine (Nishizuka, 1986).

2.4. Cholesterol, Fatty Acids and Other Neutral Lipids

Cholesterol, as well as cholesteryl ester, is also abundant in mammalian tissues. In the nervous system, they account for about 10% of the dry weight of neural tissue (Jungalwala, 1985). In human, ox, or rat liver, they account for about 5% of the total lipids (Padley, et al., 1986). The importance of cholesterol in mammalian cells is most likely for regulation and maintenance of properties such as membrane fluidity and rigidity which in turn may modulate the activities of numerous membrane bound enzymes and cellular proliferation (Bloch, 1991). However, the biological role of cholesterol in neurons is still not completely understood (Yao, 1988).

The sequence of cholesteryl biosynthesis begins with a condensation in the cytosol of two molecules of acetyl-CoA (Zubay, 1988). The enzyme β-hydroxy-β-methylglutaryl CoA synthase catalyzes the condensation of a third acetyl-CoA with β-ketobutyryl-CoA to yield β-hydroxy-β-methylglutaryl-CoA (HMG-CoA). HMG-CoA is then reduced to mevalonate by HMG-CoA reductase. The endoplasmic reticulum enzyme HMG-CoA reductase catalyzes the rate-determining step in the synthesis of cholesterol and other polyisoprenoid compounds in animal cells (Goldstein and Brown, 1990). The activity of the enzyme is subject to feedback suppression by several agents, including cholesterol derived from receptor-mediated endocytosis of plasma low density lipoprotein, oxygenated sterols and the products of the HMG-CoA reductase reaction (Goldstein and Brown, 1990). The cDNA of HMG-CoA reductase in Chinese hamster ovary cells has been cloned, and the amino acid sequence of the protein has been deduced from the nucleotide sequence of the cDNA (Chin et al., 1984)

Fatty acid is also an important component of the phospholipid bilayer, as well as a major source of fuel (Zubay, 1988). The biosynthesis of fatty acids has been found in the cytosol of many cell types and tissues. Although, it has been investigated in neurons (Gould et al., 1983a), we still do not know whether or not fatty acids can be synthesized in pure neuronal axons.

3. THESIS OBJECTIVE

Many experiments show that membrane materials such as proteins and lipids needed for axonal growth, membrane maintenance and axon regeneration are synthesized in cell bodies. These materials are then delivered to axons by anterograde transport (Alberts et al., 1989; Ledeen, 1985). However,

some basic questions, such as the following, need to be clearly answered. (i) Are only cell bodies of neurons able to synthesize the membrane materials, or can some of the materials be synthesized in axons as well as in cell bodies? (ii) Even though NGF has long been known as an indispensable neurotrophic factor for the development, survival and proliferation of sympathetic and sensory neurons (Tischler and Greene, 1975), the exact mechanism of its action is still unclear. Especially, the effect of NGF on lipid metabolism in neurons has never been addressed.

The question of whether or not lipids can be synthesized in axons has been addressed in a few papers, but the results give no clear answer (Vance, J.E. et al., 1991). For example, extruded squid axoplasm was shown to incorporate a number of precursors such as [^{3}H]choline, [^{3}H]ethanolamine, [^{3}H]serine, and *myo*-[^{3}H]inositol into phospholipids (Brunetti et al., 1979; Gould et al., 1983a). On the other hand, axon-based phospholipid synthesis from all these precursors except [^{3}H]*myo*-inositol was, at best, quite meager in mouse sciatic nerves (Gould et al., 1987). Also the result of enzyme assays suggested that cholinephosphotransferase, the terminal enzyme in the synthesis of PC along the CDP-choline pathway, was not present in axons, indicating no synthesis of PC in sciatic nerve axoplasm (Gould and Dawson, 1976; Kumara-Siri and Gould, 1980). The reason for these controversial conclusions may be that in neither of these reports were pure, intact, and living mammalian neurons used (Vance, J.E. et al., 1991), therefore they could not investigate lipid metabolism in local intact axons free from cell bodies. Thus it was difficult to distinguish the lipids produced by axons from those produced by cell bodies, or by glial cells, or by other contaminating cell types that invariably associate with axons *in vivo*.

Dr. Robert B. Campenot has developed a compartmented neuron model (Campenot, 1977; Campenot, 1982). In his model, a Teflon divider is used to partition the dish into three chambers where the cell bodies plus proximal axons are in the central chamber and the distal axons are in the left and right chambers. Therefore the cell bodies plus proximal axons can be separated from distal axons in different chambers with distinct fluid environments (see Fig. 6 and Materials and Methods for details). This model has several advantages compared with the models previously used by other researchers. First, the primary sympathetic neurons are pure and free from other cell types which are eliminated by an initial exposure of the cells to medium containing cytosine arabinoside. Cytosine arabinoside is a mitotic inhibitor which can effectively block the proliferation of other cells therefore kill these cells but leave pure neurons intact. Second, pure distal axons can be studied separately from the cell bodies. The composition of the medium in the cell body- and axon-containing chambers can be different since there is virtually no bulk-flow of culture medium or significant diffusion of even small molecules such as ions between the compartments (Campenot, 1982; Campenot, 1989).

In this thesis, the compartmented neurons and mass-cultured neurons were used and the following questions were addressed:

(1) Are axons able to synthesize proteins and lipids?

(2) What is the rate-limiting step in the synthesis of PC, the major membrane lipid, in intact neurons and in axons?

(3) Does NGF affect the biosynthesis of PC in neurons?

MATERIALS AND METHODS

1. CHEMICALS AND RADIOLABELED MATERIALS

[*Methyl*-^{3}H]choline (specific activity, 51 mCi/mmol), L-[3-^{3}H]serine (30 Ci/mmol), [1-^{3}H]ethanolamine (30.4 Ci/mmol), *myo*-[^{3}H]inositol (82 Ci/mmol), [1-^{14}C]acetate (sodium salt, 58 mCi/mmol), [*methyl*-^{3}H]*S*-adenosylmethionine (68 Ci/mmol) and L-[^{35}S]methionine (1037 Ci/mmol) were purchased from Amersham Corp. (Arlington Heights, IL). All reagents for polyacrylamide gel electrophoresis were purchased from Bio-Rad Laboratories (Cambridge, MA). Thin-layer chromatography plates (Silica Gel G60, 0.25 mm thickness) were purchased from BDH Chemicals (Canada). The standard phospholipids - PC, PE, SM and phosphatidylmonomethylethanolamine were purchased from Avanti Polar Lipids (Birmingham, AL), or from Sigma Chemical Company (St. Louis, MO). Choline, phosphocholine, CDP-choline, ethanolamine, bovine serum albumin, dithiothreitol, serine, cytidine 5'-triphosphate and Triton X-100 were from Sigma Chemical Company. Phospho[*methyl*-^{3}H]choline (5-7 mCi/mmol) was synthesized enzymatically from [*methyl*-^{3}H]choline, ATP and choline kinase as described (Vance, D.E., et al, 1981) in this laboratory. The diacylglycerol was prepared by phospholipase C digestion of egg PC (Kuksis et al., 1981). Asolectin (95% purified soybean phospholipids) was purchased from Associated Concentrates (Woodside, NY).

2. CULTURE MATERIALS AND THE PREPARATION OF NEURON CULTURES

Using previously described procedures (Campenot, 1979), superior cervica ganglia were dissected from newborn rats (Sprague-Dawleys rats supplied by the University of Alberta Farm), subjected to a combined trypsin and mechanical dissociation procedure and plated into compartmented dishes (Campenot, 1979; Campenot et al., 1991). Twenty parallel collagen tracks were formed between scratches on the floor of collagen-coated 35-mm Falcon tissue culture dish from which the dried collagen had been scraped (Fig. 6). The scratched region of the dish floor was wetted with culture medium, then a Teflon divider, which partitions the dish into three compartments, was sealed to the dish floor with silicon grease. Dissociated sympathetic neurons were plated in the center compartments as previously described (Campenot, 1979; Campenot et al., 1991).

Leibovitz's L15 medium (powder) without antibiotics (Gibco Laboratories, Grand Iland, NY) was supplemented with the prescribed additives, including bicarbonate and methylcellulose (Hawrot and Patterson, 1979). Rat serum (2.5% provided by the University of Alberta Laboratory Animal Services) and ascorbic acid were supplied only in the medium given to the center compartments containing cell bodies. Culture medium was changed every 4 days. Non-neuronal cells were eliminated by an initial exposure for 4 days to the medium containing 10 μM cytosine arabinoside, a mitotic inhibitor. Initially, all 3 chambers were supplied with 10 ng/ml 2.5 S nerve growth factor to allow cell survival in the center chamber and neurite growth into the left and right chambers. Nerve growth factor (NGF) and anti-NGF antibody were provided by the Department of Anatomy and Cell Biology in this University. In the center chamber, NGF was discontinued from the fifth day because many neurites had crossed the silicone grease barriers and entered

the left and right compartments. The neurons in the left and right chambers were continually supplied with NGF until the cells were ready for experiments. All medium contained methylcellulose unless otherwise noted (see Results section for details).

For some other experiments, mass-cultured sympathetic neurons were prepared. The cells were plated in 24-well dishes instead of the compartmented dishes. The mass-cultured neurons were treated in the same way as for the cell bodies of compartmented neurons during the first four days. After that the same medium (except cytosine arabinoside) was given to these mass-cultured neurons. Other procedures were the same as for compartmented cultures.

3. PREPARATION OF SOLUTIONS

3.1. Buffers for Sample Preparation

Concentrated Buffer A (Homogenization Buffer, X10, for 500 ml)

1500 mM NaCl	43.83 g
500 mM Tris	30.28 g
10 mM EDTA	1.86 g
0.25% NaN_3	1.25 g

This buffer was adjusted to pH 7.8 with concentrated HCl and distilled water was added to 500 ml. Stored at 4^0C. This concentrated buffer was diluted 10-fold with distilled water just before use and the pH was adjusted to 7.5 with HCl. For 500 ml buffer A (x1), 0.154 g dithiothreitol and 0.25 ml of 0.1 M phenylmethylsulfonyl fluoride (PMSF) was added.

Buffer R (for 500 ml)

10 mM Tris	0.606 g
0.25 M Sucrose	42.75 g

0.1 mM PMSF	0.5 ml of 0.1 M PMSF (in isopropanol)

Dissolved in distilled water and adjusted to pH 7.4 with HCl. Stored at 4^0C.

3.2. Buffers for SDS-Polyacrylamide Gradient Gel Electrophoresis (SDS-PAGE)

Stacking Buffer (for 100 ml)

0.5 M Tris	6.06 g
0.4% sodium dodecyl sulfate (SDS)	0.4 g

Dissolved in distilled water and adjusted to pH 6.8. Stored at 4^0C.

Protein Extraction Buffer (for 10 ml)

Stacking buffer	1.25 ml
10% SDS	2 ml
glycerol	1 ml
ß-mercaptoethanol	0.5 ml
0.1% bromophenol blue	0.2 ml

Distilled water was added to give the final volume 10 ml. Stored at 4^0C.

3.3. Solution for Silver-binding Protein Assay

Ammonical Silver Solution

4% (w/v) sodium hydroxide	1 ml
29% ammonium hydroxide	0.2 ml
Distilled water	18.6 ml

0.2 ml of 20% (w/v) silver nitrate was added dropwise to the solution. Stored in dark place at 4 ^{0}C

3.4. Solutions for CT Assay

Concentrated Stock Buffer (X10, for 500 ml)

325 mM Tris	19.7 g
225 mM NaCl	6.6 g
10 mM EDTA	1.85 g

50 mM Mg acetate 5.35 g

Dissolved in distilled water and the pH was adjusted to 7.5. Stored at 4^0C. Diluted 10-fold with distilled water before use.

Stock Mixture

Stock Buffer (x1)	800 µl
15 mM [^{3}H]phosphocholine	450 µl
135 mM CTP	100 µl

Stored in freezer.

10 mM Phosphatidylcholine:10 mM Oleate

Phosphatidylcholine (100 mg/ml)	393 µl
Oleic acid (approx. 99%)	15.8 µl

The stock phosphatidylcholine was evaporated to dryness in a rotary evaporator. Oleate and 5 ml buffer A were added. The solution was sonicated for 10 min or until clear. Stored at 4^0C. This solution was diluted 5-fold with buffer A before use. Stored at 4^0C.

Solvent System for Thin-layer Chromatography (TLC)

Methanol /0.6% NaCl/NH_4OH, 10:10:0.9 (v/v)

Color Development Reagent

0.1 g of 2',7'-dichlorofluorescein was dissolved in 100 ml methanol and kept on shelf.

3.5. Solutions for CPT Asssay

Preparation of Diacylglycerol

5 mg diacylglycerol (diolein) was placed in an Eppendorf tube, and evaporated to dryness under N_2. 0.5 ml of 50 mg/ml Asolectin solution and 5 µl of Tween 20 were added. The solution was sonicated until clear (about 5 min).

Preparation of 4 mM [^{14}C]CDP-choline

20.4 mg CDP-choline sodium salt was dissolved in 10 ml water and 20 µCi [^{14}C]CDP-choline was added to this solution. The specific activity was checked (about 0.5 µCi/µmol). Stored in a freezer.

4. INCORPORATION OF RADIOLABELED PRECURSORS INTO PHOSPHOLIPIDS AND THEIR WATER-SOLUBLE METABOLITES IN CELL BODIES AND AXONS

4.1. Incorporation of [*methyl*-^{3}H]Choline into PC and SM

Medium containing 10 µCi/ml [*methyl*-^{3}H]choline without unlabeled choline was added to either the cell body-containing compartments or the axon-containing compartments of isolated rat sympathetic neurons that had been cultured for 9 days. About 1.5 ml of medium was added to the center compartment containing the cell bodies and proximal axons, and 0.25 ml was added to each side compartment containing the axons. After incubation periods of 1 - 16 h the radioactive medium was removed, the cells were washed twice with cold PBS and cellular material was harvested from each compartment in 2 ml of methanol/water, 1:1 (v/v). The lipids were extracted from each sample by the addition of 2 ml of chloroform, so that the final ratio of chlorform/methanol/water was 2:1:1 (v/v) (Folch et al., 1957). The lower phase, containing the phospholipids, was washed twice with methanol/water, 1:1 (v/v) and evaporated to dryness under nitrogen. The lipid samples were applied to a thin-layer chromatography plate which was run in the solvent system chloroform/methanol/acetic acid/water, 50:30:8:3 (v/v) using unlabeled standard phospholipids as carriers. The spots corresponding to authentic standards of PC and SM were visualized by iodine vapor and the radioactivity was determined by liquid scintillation counting.

From the above experiments, the aqueous extract containing choline, phosphocholine and CDP-choline from the lipid extraction was retained and evaporated to dryness (Prichard and Vance, 1981). The residue was redissolved in methanol/water, 1:1 (v/v) and subjected to thin-layer chromatography,

whereby the metabolites were separated in a solvent system consisting of methanol/1.2% NaCl/ammonia, 10:10:0.9 (v/v) (Pritchard and Vance, 1981). The spots corresponding to authentic choline, phosphocholine, CDP-choline, were scraped from the plates and radioactivity was counted.

4.2. Incorporation of [1-^{3}H]Ethanolamine into PE and PC

Medium containing 10 μCi/ml [1-^{3}H]ethanolamine was added to either the cell body-containing compartments or the axon-containing compartments of isolated rat sympathetic neurons that had been cultured for 9 days. The experiment was performed as described above except that [^{3}H]ethanolamine, instead of [^{3}H]choline was used. PE and PC were isolated by thin-layer chromatography (TLC) in the solvent system chloroform/methanol/acetic acid/water, 50:30:8:3 (v/v). The spots were visualized by iodine vapor and the radioactivity was determined.

The metabolites in the aqueous phase from the lipid extraction were isolated by thin-layer chromatography in a solvent system methanol/1.2%NaCl/ammonia, 10:10:0.9 (v/v). The spots corresponding to authentic standards of ethanolamine and phosphoethanolamine were visualized by spraying the plate with 0.25% ninhydrin (dissolved in acetone). The plate was then put in an oven at 100^{0}C for 5 mins. The bands were scraped from the plates and radioactivity was determined.

4.3. Incorporation of [3-^{3}H]Serine into PS and PE

Compartmented neurons that had been cultured for 9 days were used and medium containing 250 μCi/ml [3-^{3}H]serine without unlabeled serine was added to either the cell body- or the axon-containing compartments. At the

indicated times, the medium was removed and the cells were washed twice with cold phosphate-buffered saline (PBS). The cellular material from the cell bodies and axons was separately harvested in 2 ml methanol/water, 1:1 (v/v). Then 2 ml chloroform was added so that the final composition of the mixture was chloroform/methanol/water, 2:1:1 (v/v). The lipids were extracted and isolated by thin-layer chromatography in a solvent system, chloroform/ methanol/acetic acid/water, 50:30:8:3 (v/v). The spots of phosphatidylserine and phosphatidylethanolamine were scraped and the radioactivity was measured. The total aqueous phase was also dried and the radioactivity was determined.

The incorporation of [3-^{3}H]serine into phosphatidylserine and phosphatidylethanolamine was further studied in axons alone. The cell bodies in the center chamber were cut and discarded, but the axons in both side chambers were left intact. Medium containing 500 µCi/ml [3-^{3}H]serine without unlabeled serine was added to the axon-containing compartments. At the indicated times, the medium was removed and the axons were washed twice with cold PBS. The axons were harvested in 2 ml methanol/water, 1:1 (v/v) and 2 ml chloroform was added to this mixture. The lipids were separated by thin-layer chromatography. The PS and PE bands were scraped and the radioactivity was determined.

4.4. Incorporation of *myo*-[^{3}H]Inositol into PI

Medium containing 7.5 µCi/ml *myo*-[^{3}H]inositol without unlabeled inositol was added to either the cell body-containing compartments or the axon-containing compartments of isolated rat sympathetic neurons that had been cultured for 9 days. At the indicated time points, the medium was removed

and the compartments were washed twice with cold PBS. The cellular material from each compartment was collected in 2 ml methanol/water, 1:1 (v/v) and 2 ml chloroform was added. The lower phase was washed three times with methanol/water solution. The lipids were isolated by thin-layer chromatography in choloroform/methanol/acetic acid/water, 50:30:8:3 (v/v).

The incorporation of *myo*-[^{3}H]inositol into PI in axons was further studied in axons alone. The cell bodies in the center chamber of 9-day-old neurons were removed by washing with cold PBS. Only the axons in both side chambers were left intact. Medium containing 50 μCi/ml *myo*-[^{3}H]inositol without unlabeled inositol was added to both side chambers. At the indicated times, the samples were harvested as described above. The lipids were separated by thin-layer chromatography and the radioactivity of PI was determined. The results were the average of duplicate sister cultures.

5. INCORPORATION OF [1-^{14}C]ACETATE INTO CHOLESTEROL, FATTY ACIDS AND OTHER NEUTRAL LIPIDS

Medium containing 6 μCi/ml [1-^{14}C]acetate was added to either the cell body-containing compartments or axon-containing compartments of isolated rat sympathetic neurons that had been cultured for 9 days. At the indicated times, the medium was removed and the compartments were washed twice with cold PBS. The cellular material from each compartment was collected in 2 ml methanol/water, 1:1 (v/v) and 2 ml chloroform was added to this mixture. The final composition of the mixture was chloroform/methanol/water, 2:1:1 (v/v). The lipids were extracted and isolated by thin-layer chromatography in the solvent system isopropylether/acetic acid, 96:4 (v/v). The bands of cholesterol, cholesteryl ester, diacylglycerol, triacylglycerol and fatty acids were

visualized by iodine vapor and the radioactivity was determined. The total aqueous phase was also dried and the radioactivity was measured.

6. INCORPORATION OF [^{35}S]METHIONINE INTO PROTEINS

Medium containing [^{35}S]methionine (250 µCi/ml) was added to either the cell body- or the axon-containing compartments of 9-day cultures. After 16 h the medium was removed and the cells were washed twice with cold PBS. Combined cellular material from three cultures was harvested into PBS. Bovine serum albumin (1 mg) was added to each sample as a carrier. Proteins were precipitated by the addition of an equal volume of 20% (w/v) trichloroacetic acid (10% for the final concentration). A protein pellet was obtained by centrifugation at 3,000 *x g* for 15 min. The pellet was washed three times with cold 5% trichloroacetic acid. Proteins were solubilized from the pellet by the addition of 0.5 ml of extraction buffer containing 6 M urea and 2% SDS (Vance, D.E., et al., 1984). Each sample was electrophoresed on a 3 - 15% polyacrylamide gradient gel containing 0.1% SDS. The gel was fluorographed by exposure to a Kodak film for 3 days at - 70^{0}C. Each lane of the gel contained the total protein from three dishes of cells, either from the center compartments or from the two side compartments combined.

7. ASSAY OF ENZYMES INVOLVED IN PC BIOSYNTHESIS VIA THE CDP-CHOLINE PATHWAY

7.1. Preparation of Samples

The samples from mass-cultured neurons or cell bodies and axons of compartmented neurons were harvested in cold PBS, pelleted at 100,000 x *g* (25,000 rpm, Beckman SW28 rotor) for 1 h, and homogenized in a glass-Teflon homogenizer with 50 up-and-down strokes in cold buffer A. The homogenate

was centrifuged at 500 x g for 10 min to remove unbroken cells. The supernatant was used to assay enzymes involved in PC biosynthesis via the CDP-choline pathway.

To investigate the effect of NGF on CT translocation, the supernatant from the homogenization was further pelleted at 350,000 x g_{av} (99,000 rpm, Beckman TLA-100.2 fixed angle rotor) for 15 min to separate the cytosol from the total membranes. The pellet (total membranes) was resuspended in buffer R. The total membranes and cytosol (supernatant) were kept at - 70^{0}C before use in protein and CT assays.

Rat hepatocytes prepared with an established method (Vance, J.E. and Vance, D.E., 1988) were supplied by Dr. Jean E. Vance's laboratory. The homogenate, cytosol and membranes were prepared from the hepatocytes in the same way as for samples from neurons. The activities of the enzymes in these samples from hepatocytes were assayed as a positive control.

7.2. Protein Assay

The protein content of total neurons, cell bodies and axons was determined by the silver-binding protein assay method (Krystal, 1987) when the total material for protein assay was less than 1 µg. The procedure was as follows: 50 µl of sample or standard solutions, 50 µl of 0.2% SDS solution, 20 µl of 2% Tween 20, 1 ml distilled water, 20 µl of 2.5% glutaraldehyde were added to a glass tube. The sample was vortexed for 2 sec, 200 µl of ammonical silver solution was added and vortexed for another 2 sec. Color was allowed to develop for 20 - 30 min and the reaction was stopped by addition of 40 µl of 30 mg/ml sodium thiosulfate. The optical density was measured at 420 nm. Krystal's method detects total protein of as little as 5 ng, but many reagents in buffers

commonly used interfere with the assay. Therefore, the Bio-Rad microgram protein assay (Bradford, 1976) was used when sufficient material was available (above 2 µg protein). The Bio-Rad method could detect as little as 1 µg of protein and gave an accurate and linear response over a range of 1 - 20 µg protein concentrations in buffers A and R used in the experiments.

7.3. Choline Kinase Assay

Choline kinase was assayed with [*methyl*-^{3}H]choline as described (Weinhold & Rethy, 1974). The final reaction mixture contained: 0.2 M Tris-HCl pH 8.0, 0.02 M ATP, 0.02 M $MgCl_2 6H_2O$, about 1 - 5 µg protein in cytosol plus distilled water, 0.02 M [*methyl*-^{3}H]choline chloride (specific activity 0.2 µCi/µmol)

The reaction was started by adding the radioactive choline. The mixture was incubated at 37^{0}C for 20 - 60 min and the reaction was terminated by heating the tubes at 95^{0}C for 3 min. The reaction mixture was then centrifuged at 2,000 x *g* for 10 min on a bench top centrifuge. An aliquot (30 µl) of the supernatant was spotted on a TLC plate and phosphocholine was separated from choline in a solvent system containing CH_3OH/1.2% NaCl/NH_4OH, 10:10:0.9 (v/v). The phosphocholine fraction was visualized with iodine vapor. The bands were scraped and the radioactivity in phosphocholine was determined.

7.4. CTP:Phosphocholine Cytidylyltransferase Assay

An estabilished method (Pelech et al., 1981) was used for the cytidylyltransferase assay. The procedures are as follows: about 1 - 5 µg proteins in 60 µl of enzyme sample was added to 10 µl of 2 mM PC:oleate. The

mixture was incubated at 37^{0}C for 2 min. The reaction was started by adding 30 µl stock mixture containing 15 mM [*methyl*-^{3}H]phosphocholine. The reaction mixture was incubated at 37^{0}C for 15 to 60 min. The reaction was terminated by inserting the tubes into a 90 - 95^{0}C water bath for 3 min. The reaction mixture was centrifuged at 2,000 x *g* for 5 min and 15 µl of the supernatant was applied to a silica-coated plastic TLC plate. The plate was sprayed with 0.1% of 2',7'-dichlorofluorescein and the CDP-choline bands were visualized under UV light. The bands were scraped and the radioactivity was determined.

7.5. CDP-Choline:1,2-Diacylglycerol Cholinephosphotransferase Assay

The activity of cholinephosphotransferase was assayed with an established method (Vance, J.E. and Vance, D.E., 1988). The final reaction mixture contained 1 - 5 µg total protein, 50 mM Tris-HCl (pH 7.4), 1 mM EGTA, 10 mM magnesium chloride, and 1.6 mM diacylglycerol. This mixture was preincubated at 37^{0}C for 5 min. The assay (20 min at 37^{0}C) was initiated by the addition of 10 µl of 4 mM [*methyl*-^{14}C]CDP-choline and terminated with 3 ml of $CHCl_3$:CH_3OH, 1:2 (v/v). The labeled PC product was extracted (Bligh and Dyer, 1959) and the radioactivity was determined.

R E S U L T S

1. ESTABLISHMENT OF THE CELL CULTURE SYSTEM

For compartmented neuron cultures, the sympathetic neurons of the superior cervical ganglia were dissected from newborn rats and plated in the center compartment of a collagen-coated culture dish containing a Teflon divider which formed three compartments (Fig. 6). Axons extended under the silicon grease barriers and into the left and right compartments. The neuron cultures were maintained for 9 days in the presence of 2.5 S nerve growth factor; non-neuronal cells were eliminated by treatment with a mitotic inhibitor, cytosine arabinoside, for the first 4 days (Campenot, 1979, 1982; Campenot and Draker, 1989). The center compartment contained cell bodies and proximal axons, whereas the left and right compartments contained distal axons alone. Each compartment contained a separate fluid environment since there is virtually no bulk flow of culture medium (Campenot, 1982) or significant diffusion of small molecules such as ions (Campenot and Draker, 1989) between compartments. Moreover, when a radiolabeled phospholipid precursor was supplied directly to one compartment, little or no redioactivity was detected in the medium of the other compartments during the course of the experiment. This unique three-compartment model therefore has provided a means by which, for the first time, metabolic events can be independently studied in pure mammalian axons and in the cell bodies.

For some experiments mass-cultured neurons were used. The neurons were prepared in the same way as described above, but were plated into 24-well dishes. Medium containing 2.5 S NGF (10 - 200 ng/ml NGF) was given to

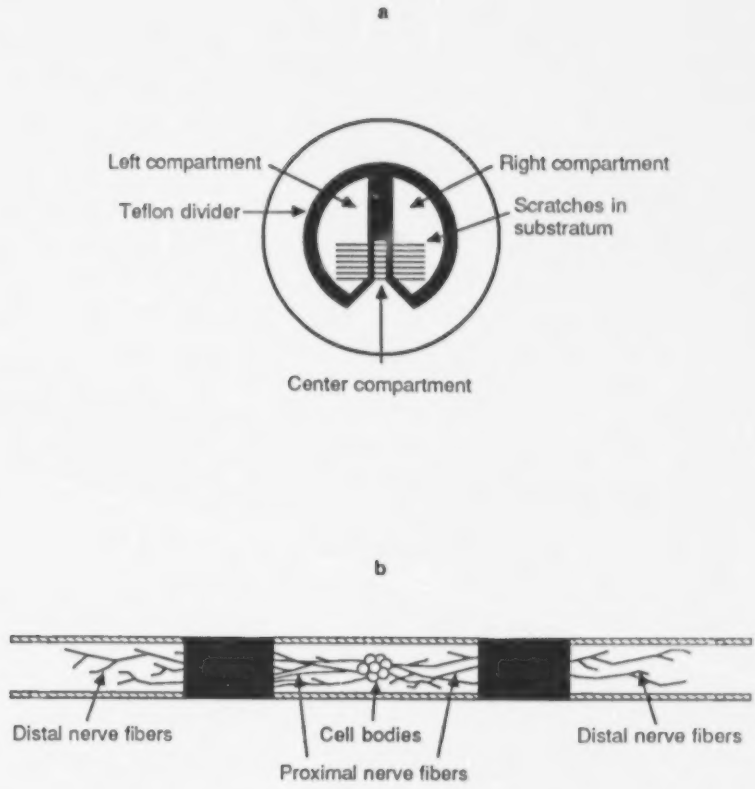


Figure 6. The Compartmented Culture Model of Neurons

This model shows (a) an entire culture and (b) the enlargement of a single tract. Dissociated neurons are plated in the center compartment (density about one ganglion per dish). Neurites extend to the left and right compartments, under silicone grease barriers and into separate fluid environments. The tracks are formed on the collagen-coated floor of a 35-mm plastic tissue culture dish between a series of parallel scratches from which the dried collagen substratum has been scraped. Each track is about 200 µm wide, the center compartments are about 1 mm wide, and the barriers are about 0.5 mm wide. One culture contains up to 20 tracks occupied by neurons. The samples from the distal neurites and from the cell bodies plus proximal neurites are harvested separately by mechanically rinsing the samples with a jet of methanol/water 1:1 (v/v) delivered with a syringe and a 22-gauge hypodermic needle. (Modified from J.E. Vance, D. Pan, D.E. Vance and R.B. Campenot, *J. Cell Biol.* 1991, **115**:1061-1068)

these cells. After 6 days the cultures were examined under the microscope and only the cells in good condition (intact neurons with full growth of neurites) were used for experiments.

2. INCORPORATION OF [*METHYL*-^{3}H]CHOLINE INTO PC AND SM IN AXONS AND CELL BODIES OF NEURONS

To investigate the sites of biosynthesis of PC and SM, two of the major lipid components of all animal cell membranes, a radiolabeled precusor [*methyl*-^{3}H]choline was added to either the center compartment containing the cell bodies and proximal axons, or to the left and right compartments containing only distal axons. After various times radiolabeled lipids were isolated from each compartment and radioactivity was measured. As expected, when [*methyl*-^{3}H]choline was added to the cell body-containing compartment, radiolabeled PC and SM were rapidly produced in that compartment. After 16 h small amounts of both radiolabeled lipids (less than 5% of the amount of radioactivity of the same lipids in the cell bodies) were apparently transported into the distal axons (Fig. 7). However, unexpectedly, when [*methyl*-^{3}H]choline was added to the axon-containing compartments alone, radioactive PC and SM rapidly appeared in the axons. No more than 15% of the radiolabeled PC and SM of the axons was present in the cell body-containing compartment even after 16 h (Fig. 7).

The major radiolabeled water-soluble precursors of PC (choline, phosphocholine and CDP-choline) in the axon- and cell body-containing compartments from the experiment described above were analyzed. The pattern of labeling of the water-soluble metabolites (Fig. 8) followed closely that of the lipid product PC. The data showed a lack of equilibration of the

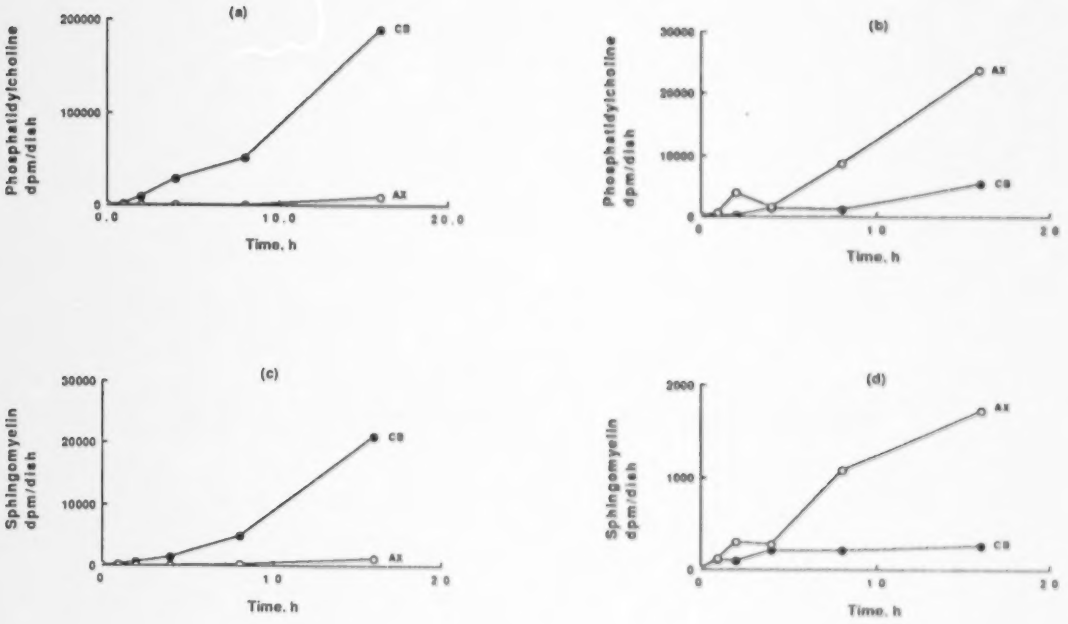


Figure 7. Incorporation of [*methyl*-^{3}H]Choline into PC and SM in Cell Bodies and Axons of Rat Sympathetic Neurons

Medium containing 10 μCi/ml of [*methyl*-^{3}H]choline was added to either the cell body-containing compartments (*panels a and c*) or the axon-containing compartments (*panels b and d*) of isolated rat sympathetic neurons that had been cultured for 9 days. At the indicated times, the medium was removed and the compartments were washed with cold PBS. The cellular material from each compartment was collected in 2 ml methanol/water, 1:1 (v/v) and then 2 ml chloroform was added. The lipids were extracted and PC (*panels a and b*) and SM (*panels c and d*) were isolated by thin-layer chromatography in the solvent system chloroform/methanol/acetic acid/water, 50:30:8:3 (v/v). The bands of PC and SM on the plates were visualized by iodine vapor and the radioactivity was determined by scintillation counting. *Solid symbols*, [^{3}H] in cell bodies (*CB*); *open symbols*, [^{3}H] in axons (*AX*). Each point is the average of two individual cultures. All data for each experiment were collected from sister cultures. The experiment was repeated three times with similar results.

water-soluble intermediates between the cell body- and axon-containing compartments, at least for the 16 h duration of the experiment. For example, even 16 h after [^{3}H]choline had been added to the axon compartments the radioactivity in choline, phosphocholine, and CDP-choline in the cell body compartment was only 9, 19, and 19%, respectively, of the radioactivity of the same intermediates in the axons.

The results in Fig. 8 demonstrate that [*methyl*-^{3}H]choline rapidly enters the cells and subsequently the [*methyl*-^{3}H]choline decreases in both the cell body- and axon-containing compartments, approximately in parallel. Radioactivity from choline subsequently appears in phosphocholine (Fig. 8), and then in PC, and SM (Fig. 7). The data presented in Figs. 7 and 8 are from one representative experiment of four that were performed. Although the amount of [*methyl*-^{3}H]choline used in the four experiments was not identical, and there was some variation in the time points at which samples were harvested, basically similar results were obtained from each experiment.

In rat liver the enzyme CTP:phosphocholine cytidylyltransferase is, under many metabolic conditions, the rate-limiting enzyme for PC biosynthesis (Vance, D.E., 1990). In the neuronal cultures used in the present experiments the amount of radioactivity in CDP-choline was at all times much lower than that in phosphocholine. For example, 8 h after the addition of [*methyl*-^{3}H]choline to the cell body-containing compartment the radioactivity in CDP-choline in the cell body compartment was 3,860 dpm/dish, whereas the radioactivity in phosphocholine was 72,720 dpm/dish. Similarly, 8 h after [*methyl*-^{3}H]choline had been added to the axon compartment the radioactivity in CDP-choline in the axon compartment was 1,950 dpm/dish, and in phosphocholine was 24,650 dpm/dish. This suggests that the "bottleneck" in PC

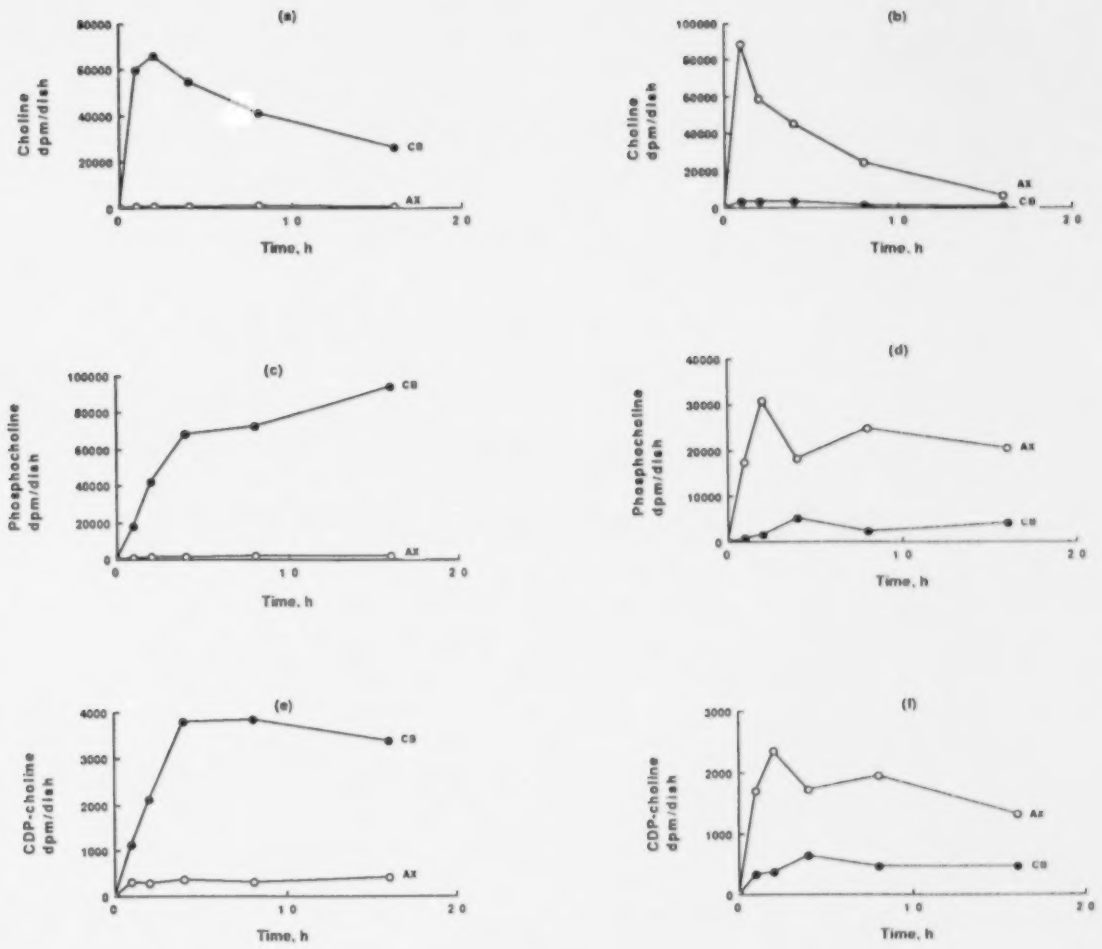


Figure 8. Incorporation of [*methyl*-^{3}H]Choline into Water-soluble Metabolites in Cell Bodies and Axons

From the experiments depicted in Fig. 7, the metabolites in the aqueous phase from the lipid extraction were isolated by thin-layer chromatography in the solvent system methanol/1.2% NaCl/ammonia, 10:10:0.9 (v/v). The spots corresponding to authentic standards of choline (*panels a and b*), phosphocholine (*panels c and d*) and CDP-choline (*panels e and f*) were scraped from the plates and radioactivity was measured. Each point is the average of two individual cultures. Three additional similar experiments were performed with similar results. All data for each experiment were collected from sister cultures. *Solid symbols*, [^{3}H] in cell bodies (*CB*); *open symbols*, [^{3}H] in axons (*AX*).

biosynthesis via the CDP-choline pathway in sympathetic neurons is at the stage of production of CDP-choline from phosphocholine. Therefore, CT is most likely the rate-limiting enzyme in this pathway.

These data demonstrate that, contrary to the generally-accepted belief that cell bodies supply all the membrane lipids for axonal growth, PC and SM were efficiently biosynthesized in axons. In addition, there was not a rapid intracellular transport of the radiolabeled phospholipids, or their water-soluble precursors, from axons and to cell bodies, and vice versa.

3. INCORPORATION OF [1-^{3}H]ETHANOLAMINE INTO PE AND PC FROM PE METHYLATION PATHWAY IN BOTH AXONS AND CELL BODIES OF NEURONS

Although the majority of PC in all mammalian cells is synthesized by the CDP-choline pathway (Vance, D.E., 1990), PC can be synthesized by an alternative route in which PE is methylated to PC by 3 consecutive PE *N*-methylation reactions (Vance and Ridgway, 1988). The results in Fig. 9 show that PE (*panels a and b*) is synthesized from [1-^{3}H]ethanolamine in both the cell body- and axon-containing compartments. Similarly, the methylation of [^{3}H]-labeled PE to PC occurs in each of the compartments containing the cell bodies and the axons (Fig 9, *c and d*). The data presented in Fig. 9 are from one experiment that is representative of two additional similar experiments. Although there were some variations in the amounts of radioactivity added to the cells and the time points at which the samples were collected, the conclusions were the same from all three experiments. For example, the increased incorporation of [1-^{3}H]ethanolamine into PC between 8 and 16 h was apparent in all experiments. In the two experiments for which the data are not

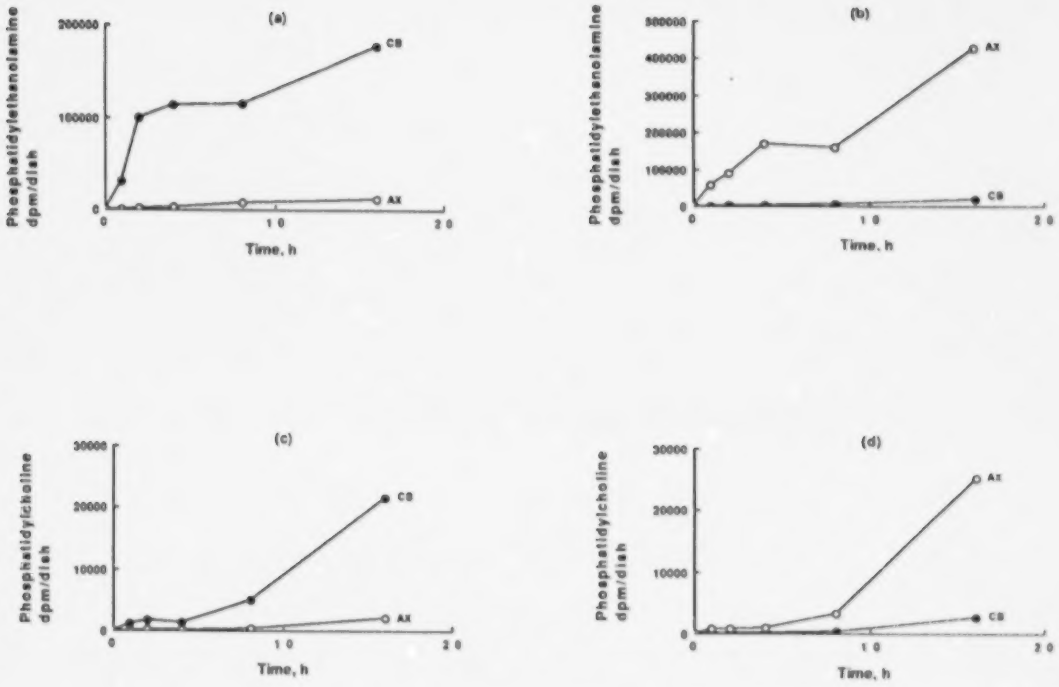


Figure 9. Incorporation of [1-^{3}H]Ethanolamine into PE and PC of Cell Bodies and Axons

Medium containing [1-^{3}H]ethanolamine (10 μCi/ml) was added to either the cell body-containing compartments (*panels a and c*) or the axon-containing compartments (*panels b and d*) of isolated rat sympathetic neurons. The experiment was performed as described in Fig. 7 except that [^{3}H]ethanolamine, instead of [^{3}H]choline was used. PE (*panels a and b*) and PC (*panels c and d*) were isolated by thin-layer chromatography. *Solid symbols*, [^{3}H] in cell bodies (*CB*); *open symbols*, [^{3}H] in axons (*AX*). Each point is the average of two individual cultures. All data for each experiment were collected from sister cultures. Two additional similar experiments were performed with similar results.

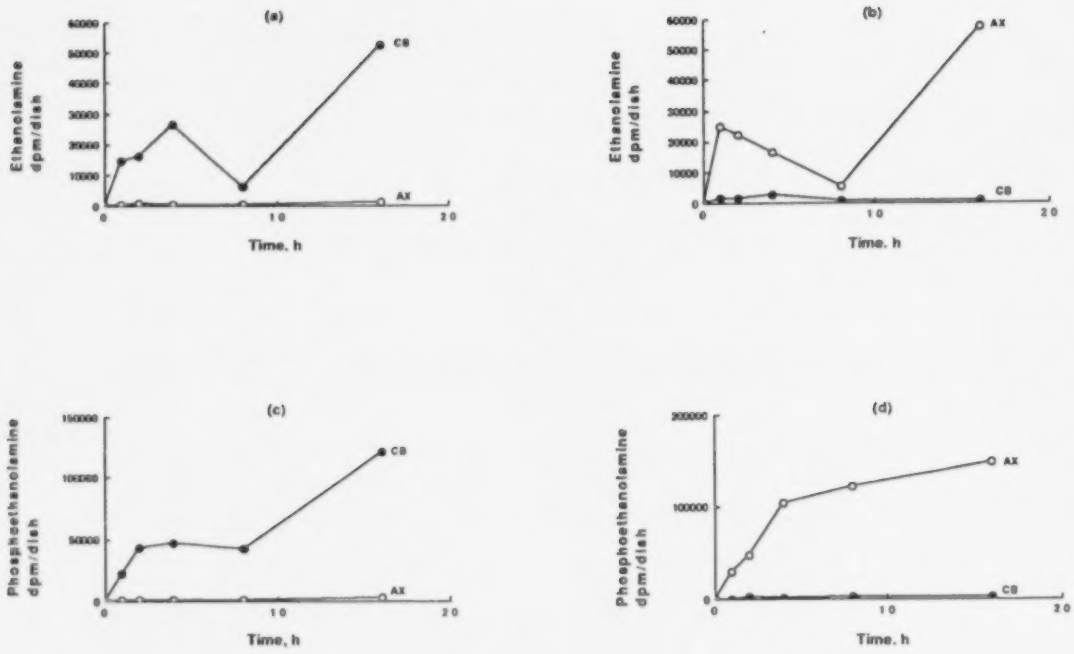


Figure 10. Incorporation of [1-^{3}H]Ethanolamine into Water-soluble Metabolites of Cell Bodies and Axons

From the experiments depicted in Fig. 9 the metabolites in the aqueous phase from the lipid extraction were isolated by thin-layer chromatography in a solvent system methanol/1.2%NaCl/ammonia, 10:10:0.9 (v/v). The spots corresponding to authentic standards of ethanolamine (*panels a and b*) and phosphoethanolamine (*panels c and d*) were visualized by spraying the plate with 0.25% ninhydrin in acetone. The bands were then visualized by putting the plate in an oven at 100^{0}C for 5 min. The bands were scraped from the plate and radioactivity was measured. *Solid symbols*, [^{3}H] in cell bodies (*CB*); *open symbols*, [^{3}H] in axons(*AX*).

Table 3. Amount of Protein of Cell Bodies and Axons from 9-day-old Neurons

For each assay, the samples were collected from the cell body- or axon-containing compartment in cold PBS from 10 dishes of compartmented neurons cultured for 9 days. The materials were pelleted by centrifugation at 100,000 x g_{av} (25,000 rpm, Beckman SW28 rotor) for 1 h. The pellets were resuspended in cold 50 mM sucrose/50 mM Hepes buffer and homogenized with a glass-Teflon homogenizer with 50 up-and-down strokes. The total proteins in homogenate were determined by a silver-binding protein assay method (Krystal, 1987).

Sample	Content (X ± S.D.) μg/dish
*Cell bodies	0.82 ± 0.24
Axons	0.33 ± 0.11

*Sample was collected from the cell body-containing compartment.

given, the increase in radiolabeled PC in the cell body compartment during this time period was 2.1- and 4.9-fold, whereas in the axon compartments was 4.4- and 6.7-fold. By 16 h of incubation with the radiolabeled precursor, 6 and 11% of the PE in the axon and cell body compartments, respectively, had been methylated to PC. This is the most efficient conversion of PE to PC that has been reported for cells other than hepatocytes (Vance and Ridgway, 1988).

The water-soluble precursors of PE were isolated by thin-layer chromatography from the incubations described in the legend to Fig. 9. The distribution and labeling patterns of two of the intermediates of PE biosynthesis from ethanolamine in the cell body- and axon-containing compartments (i.e., ethanolamine and phosphoethanolamine) are presented in Fig. 10. As was observed for the [*methyl*-^{3}H]choline metabolites (Fig. 8), the [^{3}H]ethanolamine-labeled intermediates were largely confined to the compartments to which the [1-^{3}H]ethanolamine had been added.

Figs. 9 and 10 show that in axons, as well as in cell bodies, PE is actively synthesized from [1-^{3}H]ethanolamine and that the PE is efficiently methylated to PC. The experiments using [*methyl*-^{3}H]choline (Figs. 7 and 8) and [1-^{3}H]ethanolamine (Figs. 9 and 10) in combination demonstrate that axons, independently of cell bodies, have the capacity for synthesis of the three major phospholipids of axonal membranes - PC, PE, and SM.

Table 3 shows the protein content in cell bodies and axons of 9-day-compartmented neurons. The protein content in the cell body-containing compartment is 0.82 ± 0.24 µg/dish and in the 2 axon compartments combined is 0.33 ± 0.11 µg/dish. As noted in Figs. 7 and 9, the incorporation of [*methyl*-^{3}H]choline into PC and SM, and of [1-^{3}H]ethanolamine into PE and PC is very active in axons. If the results were expressed in dpm/µg protein, instead of

dpm/dish, the axonal contribution to phospholipid biosynthesis is even greater.

4. THE MAJOR ENZYMES INVOLVED IN PC BIOSYNTHESIS VIA THE CDP-CHOLINE PATHWAY ARE PRESENT IN BOTH AXONS AND CELL BODIES

Choline kinase, cytidylyltransferase and cholinephosphotransferase are three enzymes involved in PC biosynthesis via the CDP-choline pathway (Fig. 4) (Vance, D.E., 1990). To obtain further insight into PC biosynthesis in neurons, the *in vitro* activities of these enzymes were examined in both the cell body- and the axon-containing compartments, respectively. The materials were harvested separately from the cell body- and the axon-containing compartments of compartmented neurons and homogenized with a glass-Teflon homogenizer for 40 strokes. The unbroken cells were removed by centrifugation at 500 x *g* for 10 min. The homogenate was used for the enzyme assays. Rat hepatocytes (supplied by Dr. Jean Vance's laboratory) (Vance, J.E. and Vance, D.E., 1988) were also harvested and homogenized in the same way as for neurons. The enzyme activities were assayed in the hepatocyte homogenate as a positive control.

Table 4 shows that all three enzymes of PC biosynthesis via the CDP-choline pathway are present in both the materials derived from cell body- and axon-containing compartments. The specific activities of these enzymes in cell bodies and axons were similar to those in hepatocytes. The specific activity of CK was the highest and that of CT was the lowest of three enzymes in the pathway.

Table 4. Assay of the Major Enzymes Involved in PC Biosynthesis via the CDP-Choline Pathway in Cell Bodies and Axons of Neurons

Each sample was prepared from 20 dishes of 9-day-old compartmented neurons. The cell bodies and axons were harvested in cold PBS separately, then pelleted by centrifugation at 100,000 x g_{av} (25,000 rpm, Beckman SW28 rotor) for 1 h. The pellets were homogenized in a glass-Teflon homogenizer with 50 up-and-down strokes in buffer A. The unbroken cells were removed by centrifugation at 500 x g for 10 min. The enzyme activities in homogenates were determined as described in the Methods section. A homogenate was also prepared from cultured rat hepatocytes and the enzymes were assayed for a positive control. In each enzyme assay the total amount of protein was about 5 μg. The data were from 3 separate cell preparations.

Enzyme*	Specific Activity (X ± S.D.) nmol/min/mg protein		
	Cell bodies	Axons	Hepatocytes
CK	4.69 ± 1.72	8.77 ± 3.30	4.73 ± 0.18
CT	0.69 ± 0.27	0.65 ± 0.13	0.46 ± 0.06
CPT	1.13 ± 0.18	0.93 ± 0.08	1.00 ± 0.15

* CK, *choline kinase;* CT, *CTP:phosphocholine cytidylyltransferase;* CPT, *CDP-choline:1,2-diacylglycerol cholinephosphotransferase.*

5. INCORPORATION OF [3-^{3}H]SERINE INTO PS AND PE IN BOTH AXONS AND CELL BODIES OF NEURONS

The biosynthesis of PS, and PE from PS were investigated by the addition of 250 μCi/ml [^{3}H]serine, a radiolabeled precursor of these two lipids, to both cell bodies and axons. As expected, [3-^{3}H]serine was incorporated into PS and PE in the cell bodies (Fig. 11, *panels a and c*). Although it seems that [3-^{3}H]serine was also incorporated into PS and PE in the axons, the radioactivity in PS and PE bands was low (below 300 dpm/dish after 16 h, see Fig. 11, *panels b and d*). The results in Fig. 11, *panels g and h* indicate that [3-^{3}H]serine did enter the cell bodies and axons.

Because of the low level of radioisotope incorporation in the above experiment, PS biosynthesis was further studied in axons alone using a larger amount of [^{3}H]serine. The cell bodies in the center chamber of compartmented neurons cultured for 9 days were removed by washing with cold PBS and axons in the 2 side chambers were left intact. Medium containing 500 μCi/ml [3-^{3}H]serine was added to these two chambers. At the indicated times, the radioactivity in PS and PE was determined. Fig. 12 shows that the dpm/dish in the PS and PE bands was 2,694 and 1,721, respectively, at 4 h and 3,130 and 1,910 at 8 h, respectively. Therefore, we conclude that PS, and PE derived from PS, were synthesized locally in the axons.

6. INCORPORATION OF *MYO*-[^{3}H]INOSITOL INTO PI IN BOTH AXONS AND CELL BODIES OF NEURONS

To investigate whether or not PI could also be synthesized in axons, medium containing 7.5 μCi/ml *myo*-[^{3}H]inositol was added into either the cell

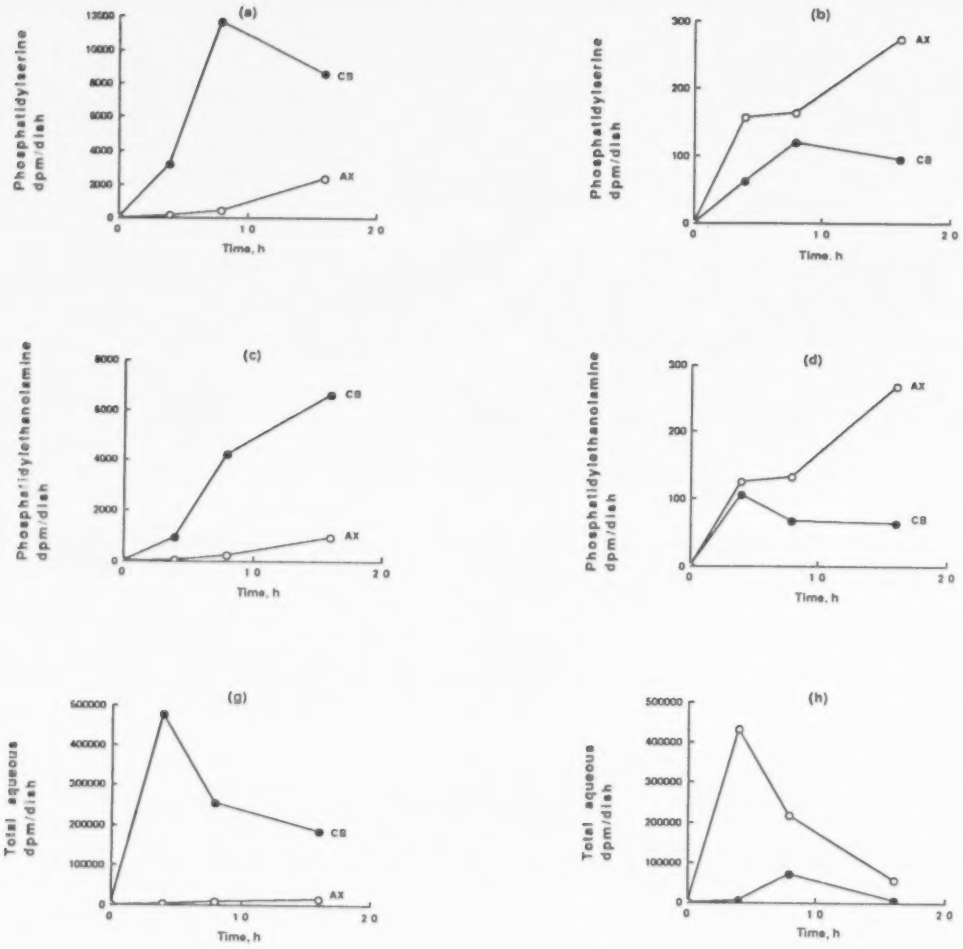


Figure 11. Incorporation of [3-^{3}H]Serine into PS and PE in The Cell Bodies and Axons of Neurons

Medium containing 250 μCi/ml [3-^{3}H]serine without unlabeled serine was added to either the cell body-containing compartments (*panels a, c and d*) or the axon-containing compartments (*panels b, d and h*) of isolated sympathetic neurons. At the indicated times, the medium was removed and the cells were washed twice with cold PBS. The materials from the cell bodies and axons were harvested in 2 ml methanol/water, 1:1 (v/v) separately. Then 2 ml chloroform was added. The lipids were extracted and isolated by thin-layer chromatography in the solvent system chloroform/methanol/acetic acid/ water, 50:30:8:3 (v/v). The spots of PS and PE were visualized by iodine vapor. The bands were then scraped and the radioactivity was mesaured. The total aqueous phase was also dried and the radioactivity was determined (*panels g and h*). *Solid symbols*, [^{3}H] in cell bodies (*CB*); *open symbols*, [^{3}H] in axons (*AX*).

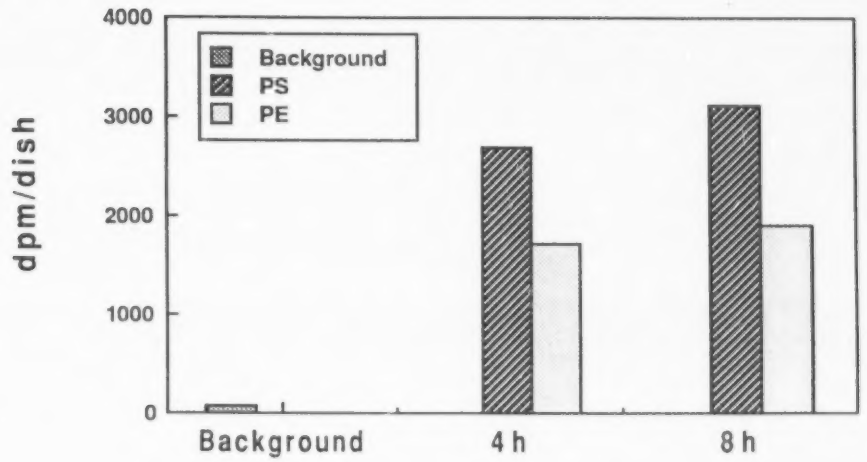


Figure 12. Incorporation of [3-^{3}H]Serine into PS and PE in The Axons after The Cell Bodies were Removed from Center Chamber

The cell bodies of 9-day old neurons were removed from the center chamber, but the axons in both side chambers were left intact. Medium containing 500 μCi/ml [3-^{3}H]serine was added to the axon-containing compartments. At the indicated times, the medium was removed and the axons were washed twice with cold PBS. The axons were harvested in 2 ml methanol/water, 1:1(v/v) and 2 ml chloroform was added to this mixture. The final composition of the mixture is chloroform/methanol/water, 2:1:1 (v/v). The lipids were separated by thin-layer chromatography and the radioactivities in PS and PE were determined. The results were obtained from the average of duplicate sister dishes.

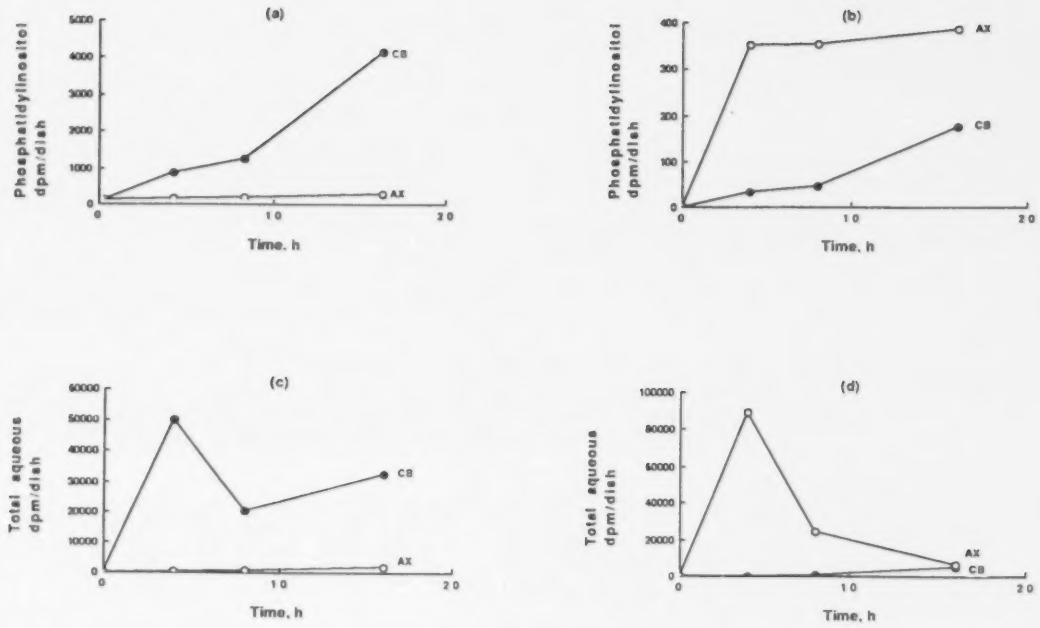


Figure 13. Incorporation of *myo*-[^{3}H]Inositol into PI in Cell Bodies and Axons of Neurons

Medium containing 7.5 μCi/ml *myo*-[^{3}H]inositol without unlabeled inositol was added to either the cell body-containing compartment (*panels a and c*) or the axon-containing compartment (*panels b and d*) of isolated rat sympathetic neurons that had been cultured for 9 days. At the indicated time points, the medium was removed and the compartments were washed twice with cold PBS. The cellular material from each compartment was collected in 2 ml methanol/water, 1:1 (v/v) and 2 ml chloroform was added. The lipids were isolated by thin-layer chromatography in the solvent system chloroform/ methanol/acetic acid/water, 50:30:8:3 (v/v). The bands of PI were scraped from the plates and the radioactivity was measured. The total aqueous phase was also blown to dryness and the radioactivity was determined. *Solid symbols*, [^{3}H] in cell bodies (*CB*); *open symbols*, [^{3}H] in axons (*AX*). The experiment was repeated two more times with similar results.

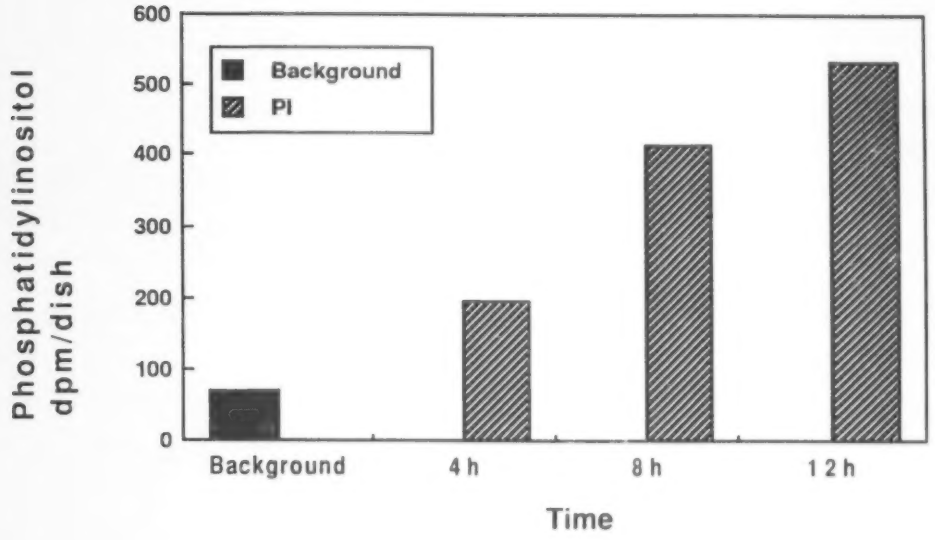


Figure 14. Incorporation of *myo*-[^{3}H]Inositol into PI in Axons after The Cell Bodies were Removed from The Center Compartment

The cell bodies in the center chamber of 9-day-old neurons were removed and the axons in both side chambers were left intact. Medium containing 50 μCi/ml *myo*-[^{3}H]inositol without unlabeled inositol was added into both side chambers. At the indicated times, the samples were harvested as described in Fig. 13. The lipids were separated by thin-layer chromatography and the radioactivity in PI was determined. The results were the average of duplicate sister cultures.

body- or the axon-containing compartments of neurons cultured in the compartment model for 9 days.

Fig. 13 shows that *myo*-[^{3}H]inositol penetrated into the inside of cell bodies and axons (*panels c and d*). Sixteen h after addition of *myo*-[^{3}H]inositol to the cell body-containing compartment about 4,000 dpm/dish was detected in PI in the material derived from cell body-containing compartment, whereas, about 160 dpm/dish was found in the PI in the material derived from axon-containing compartment (*panel a*). When radioisotope was added to the axon-containing compartments about 400 dpm/dish was detected in the PI in the material derived from axon-containing compartment, and about 180 dpm/dish in the PI in the materials derived from the cell body-containing compartment (Fig. 13, *panel b*).

PI biosynthetic activity was further investigated in axons alone. The cell bodies in the center chamber of 9-day old neurons were removed and the axons in the 2 side chambers were left intact. Medium containing 50 μCi/ml *myo*-[^{3}H]inositol was added into the 2 side chambers. At the indicated times, the samples were harvested and the radioactivity in PI was determined. Fig. 14 shows that *myo*-[^{3}H]inositol was incorporated in a time-dependent manner into PI in the axons.

7. INCORPORATION OF [1-^{14}C]ACETATE INTO CHOLESTEROL, FATTY ACIDS AND OTHER NEUTRAL LIPIDS IN CELL BODIES

Figs. 15 and 16 show that the incorporation of [^{14}C]acetate into cholesterol, cholesterol esters and other neutral lipids could only be detected in the cell body-containing compartment (Fig. 15, *panels a and b*; Fig. 16,

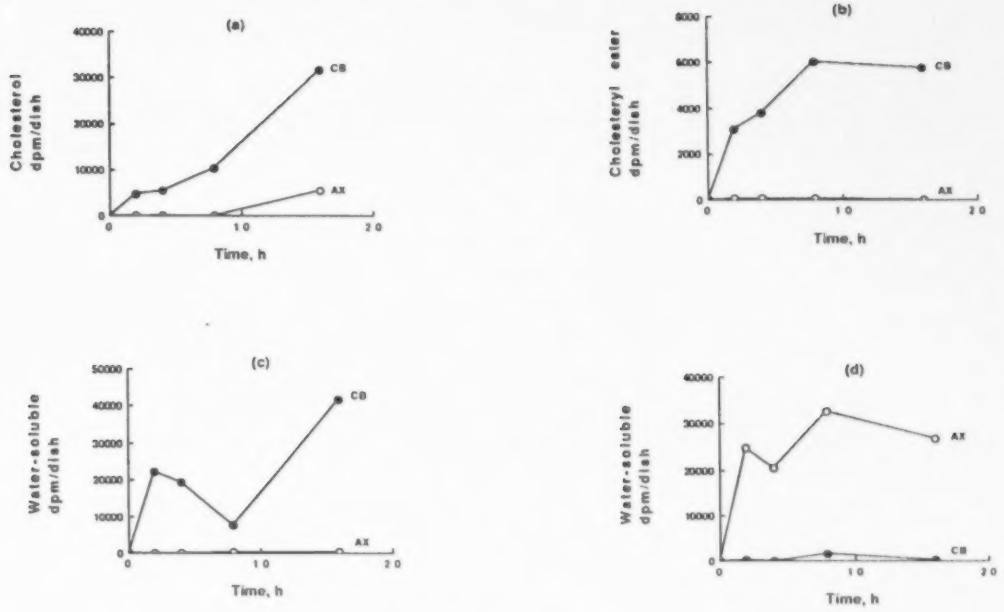


Figure 15. Incorporation of [1-^{14}C]Acetate into Cholesterol and Cholesteryl Esters in Cell Bodies

Medium containing 6 μCi/ml [1-^{14}C]acetate was added to either the cell body-containing compartments (*panels a, b and c*) or the axon-containing compartments (*panel d*) of isolated rat sympathetic neurons that had been cultured for 9 days. At the indicated times, the medium was removed and the compartments were washed twice with cold PBS. The cellular material from each compartment was collected in 2 ml methanol/water, 1:1 (v/v) and 2 ml chloroform was added. The lipids were isolated by thin-layer chromatography in the solvent system isopropyl ether/acetic acid, 96:4 (v/v). The bands of cholesterol and cholesteryl ester were visualized by iodine vapor and the radioactivity was determined. The total aqueous phase was also dried and the radioactivity was measured. *Solid symbols*, [^{14}C] in cell bodies (*CB*); *open symbols*, [^{14}C] in axons (*AX*). Each point is the average of two individual cultures. All data for each experiment were collected from sister cultures. The experiment was repeated two times with similar results.

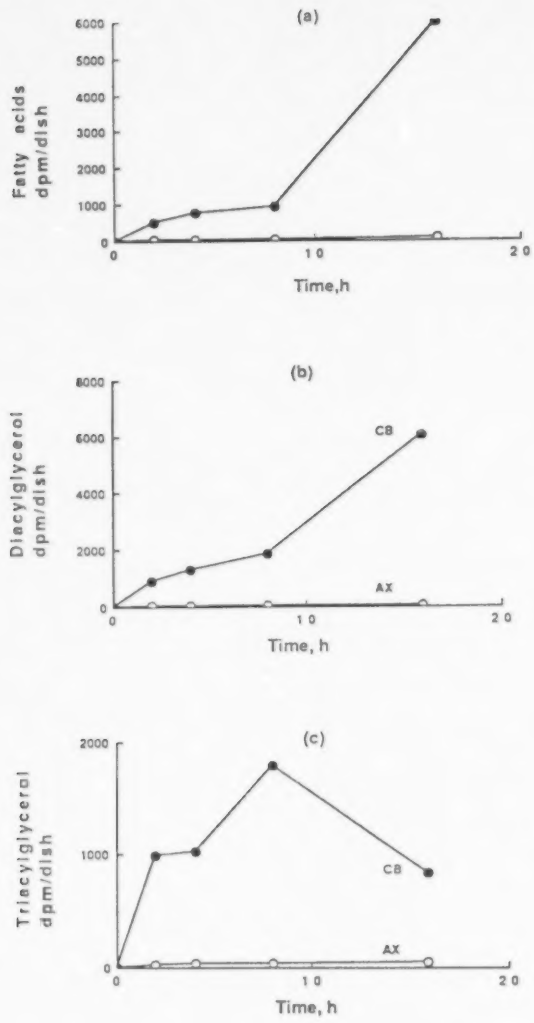


Figure 16. Incorporation of [1-^{14}C]Acetate into Fatty Acids, Diacylglycerol and Triacylglycerol

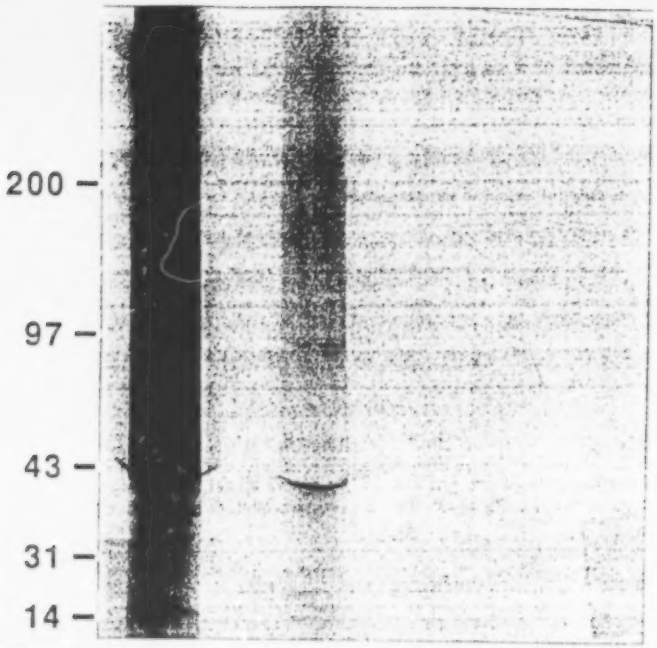
From the experiment depicted in Fig. 15, fatty acids (*panel a*), diacylglycerol (*panel b*) and triacylglycerol (*panel c*) from the cell body-containing compartment were obtained by thin-layer chromatography. The spots of these lipids were visualized by iodine vapor. The bands were scraped and the radioactivity was determined. *Solid symbols*, [^{14}C] in cell bodies (*CB*); *open symbols*, [^{14}C] in axons (*AX*).

panels a, b and c). After 16 h some cholesterol (5,420 dpm/dish) was transported to axons from the cell body-containing compartment (Fig. 15, *panel a*).

Axons apparently did not synthesize any of these lipids. At times up to 16 h after the addition of [^{3}H]acetate to the axon-containing compartments the radioactivity in all the corresponding lipids of axons was no more than that of the blank (less than 60 dpm/dish). [^{14}C]Acetate did penetrate inside the cell bodies and the axons since large amounts of radioactivity were found in the aqueous phase of both cell bodies and axons (Fig. 15, *panels c and d*). Therefore, there is no evidence that axons can synthesize cholesterol, cholesteryl esters, fatty acids, diacylglycerol, or triacylglycerol.

8. INCORPORATION OF [^{35}S]METHIONINE INTO PROTEINS IN CELL BODIES OF NEURONS

Since ribosomes have been detected only in cell bodies but not in axons (Alberts, et al., 1989), we would expect that protein synthesis would occur only in cell bodies. Therefore protein synthesis was investigated in the three compartmented neuron model. L-[^{35}S]Methionine was added to either the cell body-containing compartment or to the axon-containing compartments for 16 h at which time cellular material was harvested from each compartment. Proteins were isolated and subjected to electrophoresis on a 3 - 15% polyacrylamide gradient gel containing 0.1% SDS. The gel was fluorographed and the radiolabeled proteins are illustrated in Fig. 17. As expected, when [^{35}S]methionine was added to the cell body compartment radiolabeled proteins were observed in that compartment, with some radiolabeled proteins being



compartment	CB	AX	CB	AX
[^{35}S] added	CB	CB	AX	AX

Figure 17. Incorporation of [^{35}S]Methionine into Neuronal Proteins

Medium containing [^{35}S]methionine (250 μCi/ml) was added to either the cell body-containing compartment or the axon-containing compartments of isolated rat sympathetic neurons. After 16 h the medium was removed and the compartments were washed twice with cold PBS. Cellular material from three cultures combined was harvested from the individual compartments. After the addition of albumin as a protein carrier the proteins were precipitated with a final concentration of 10% TCA. The pellet was washed twice with 5% TCA. Proteins were solubilized by heating the pellet in an extraction buffer containing 6M urea and 2% SDS. Each sample was subjected to electrophoresis on a 3-15% gradient polyacrylamide gel containing 0.1% SDS. The gel was fluorographed by exposure to a photographic plate for 3 days at -70^{0}C. The numbers to the left of the gel represent molecular masses (x 10^{-3} kD) of standard proteins. The protein bands were cut from the gel and the radioactivity of the four lanes was determined. From left to right, the total radioactivity was $2.5x10^4$, $2.5x10^3$, 204 and 169 dpm/lane, respectively. The experiment was repeated twice with similar results. Each lane contained the total protein from three dishes of cells, either from the center compartments or from the two side compartments combined.

apparently transported into the axons. In contrast, when [^{35}S]methionine was added to the axon compartments alone, no radiolabeled proteins were detected either in the axon- or cell body-containing compartments. Therefore protein synthesis could be detected only in cell bodies.

9. PULSE-CHASE STUDIES OF INTACT NEURONS, CELL BODIES AND AXONS

In liver cells, under most metabolic conditions, the enzyme CT catalyses the rate-limiting step of PC biosynthesis (Vance, D.E., 1989a; Vance, D.E., 1989b). Thus, the rate of PC biosynthesis can be controlled by factors which either increase or decrease the activity of this enzyme.

A major objective of this thesis is to determine which enzyme regulates the rate of PC biosynthesis in intact neurons. Pulse-chase studies were therefore performed. Medium containing 10 μCi/ml [*methyl*-^{3}H]choline was added to neurons cultured in 24-well plates rather than in the compartment cultures. Similarly, the rate-limiting step of PC biosynthesis in axons and cell bodies was examined independently. Medium containing 20 μCi/ml [*methyl*-^{3}H]choline was added to the chambers of cell bodies or axons in the compartmented cultures of neurons. Incorporation of radioactivity into cellular choline, phosphocholine and CDP-choline, as well as into PC, was measured by scintillation counting of the TLC bands. We hypothesized that if CT were the rate-limiting enzyme in the biosynthetic pathway as has been observed in hepatocytes (Vance, D.E., 1989a) and other cell types (Vance, D.E., 1989a), then the radioactivity in CDP-choline would be expected to be very low compared to that in phosphocholine (Vance, D.E., 1989a; Vance, D.E., 1989b).

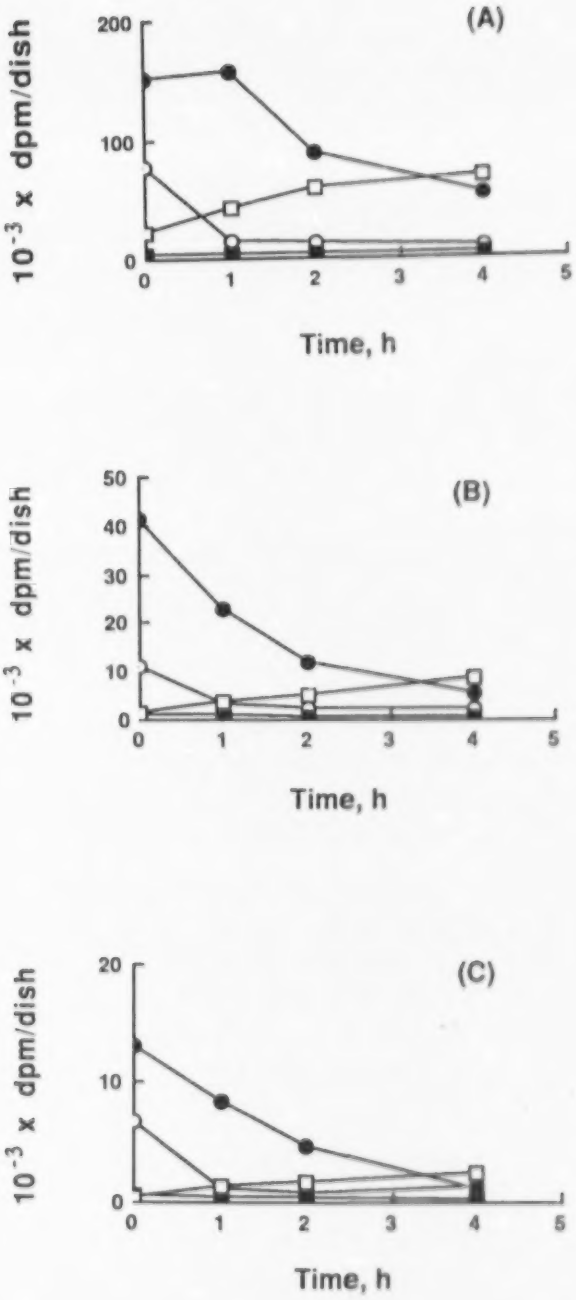


Figure 18. Pulse-chase Study in Whole Neurons, Cell Bodies and Axons with [*methyl*-^{3}H]Choline

Medium containing 10 μCi/ml [*methyl*-^{3}H]choline without unlabeled choline was added to neurons that had been cultured for 6 days in 24-well culture dishes (*panel A*). After 1 h, the medium was removed and the cells were washed twice with cold PBS. Then the chase medium containing 28 μM unlabeled choline was added. At the indicated times, the cells were harvested in 2 ml methanol/water, 1:1 (v/v) and 2 ml chloroform was added. The lipids were isolated by thin-layer chromatography. For the parallel study in cell bodies (*panel B*) and axons (*panel C*), medium containing 20 μCi/ml [*methyl*-^{3}H]choline was added to the chambers. Other procedures were the same as for mass-cultured neurons. *Solid squares*, CDP-choline; *open squares*, PC; *solid circles*, phosphocholine; *open circles*, choline. The experiment was repeated with a similar result.

The results in Fig. 18 show that in the neurons cultured in the 24-well dishes the reaction catalyzed by CT was the slowest step and that the radioactivity in CDP-choline was very low compared to that in phosphocholine. This means that the "bottleneck" in the PC biosynthetic pathway in neurons was at the stage of production of CDP-choline from phosphocholine. Therefore, CT is also the rate-limiting enzyme of PC biosynthesis in the intact neurons (Fig. 18, *panel A*). A similar result was observed in the cell bodyies (Fig. 18, *panel B*) and axons (Fig. 18, *panel C*) of compartmented cultures of neurons.

10. EFFECT OF NERVE GROWTH FACTOR ON THE INCORPORATION OF [^{3}H]CHOLINE INTO PC

NGF promotes axonal growth in PC12 cells (Greene and Tischler, 1982) and in rat sympathetic neurons (Campenot, 1977). We postulated that one likely response of these cells to NGF was an increase in the rate of PC biosynthesis, the major membrane phospholipid. Therefore, the effect of NGF on PC biosynthesis was investigated. Neurons were cultured in medium containing 10 ng/ml NGF for 6 days. Then the fresh medium without NGF was added. After 24 h, for one group of cells the medium was changed to medium without NGF; the other group of cells was given medium containing 200 ng/ml NGF. The effect of NGF on PC biosynthesis was studied by incubation of the cells for 0, 6, 12 or 24 h in these two concentrations of NGF. After these times, fresh medium containing 10 μCi/ml [*methyl*-^{3}H]choline and the same concentration of NGF was added for 1 h. The results shown in Fig. 19, *panel B* suggest that the increase of radioactivity in phosphocholine + CDP-choline might be caused by NGF. Fig. 19, *panel C* shows that the incorporation of [^{3}H]choline into PC was

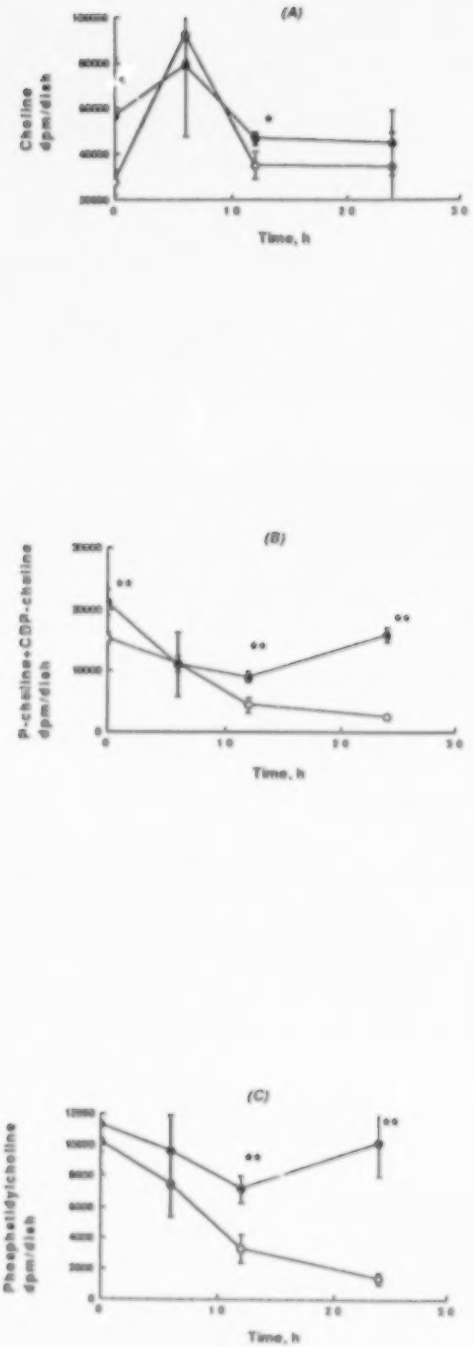


Figure 19. Effect of NGF on PC Biosynthesis in Intact Neurons

Neurons were cultured in medium containing 10 ng/ml NGF in multiwell dishes for 6 days. The medium was replaced with medium without NGF. After 24 h, the medium was changed: for one group, medium without NGF was added; for the other group, medium containing 200 ng/ml NGF was added. At times from 0 to 24 h pulse medium containing 10 μCi/ml [^{3}H]choline without unlabeled choline was added. After 1 h, the pulse medium was removed. The cells were washed twice with cold PBS and harvested in methanol/ water, 1:1 (v/v). The lipids and water-soluble intermediates were separated by thin-layer chromatography. The bands were visualized by iodine vapor and the radioactivity of choline (*panel A*), phosphocholine plus CDP-choline (*panel B*) and PC (*panel C*) was measured. *Solid circles*, + 200 ng/ml NGF; *open circles*, no NGF. *$0.01 < p \leq 0.05$; **$p \leq 0.01$.

significantly lower for the cells grown in the medium without NGF compared to the cells grown in the presence of NGF. Although preliminary, these data suggest that NGF might increase the rate of incorporation of [^{3}H]choline into PC. The difference between the label in phosphocholine + CDP-choline in the two groups of cells at 0 h in *panel B* might be caused by the increased influx of labeled choline as seen in Fig. 19, *panel A*.

One possible mechanism by which PC biosynthesis could be increased by NGF would be that NGF caused the translocation of CT from cytosol to membranes, and consequently increased the rate of PC biosynthesis.

This postulate was tested by examination of the effect of NGF on CT activity in the cytosol and membranes of neurons. Mass-cultured neurons were grown in a minimal concentration of NGF (10 ng/ml) for 6 days and subsequently were treated in one of two ways: for the control group of cells, fresh medium containing 10 ng/ml NGF was added; for the other group of neurons, medium containing 200 ng/ml NGF was added. After 2 h and 24 h, the activity of CT was assayed. Table 5 shows that there was no difference in the activity of CT in either the membranes or the cytosol of cells treated with the 2 different concentrations of NGF: most enzyme activity was present in the cytosol, presumably as an inactive reservoir.

The effect of NGF on PC biosynthesis was further examined. Neurons were cultured in medium containing 200 ng/ml NGF for 9 days. The cells were divided into two groups and two different conditions were compared. In one group of neurons, medium containing 200 ng/ml NGF was added; for the other group of cells, medium lacking NGF but containing 24 nM anti-NGF antibody was added. The antibody was used to neutralize any residual NGF that may not have been removed when the medium was changed (Campenot, 1991). After 6 h

Table 5. The Effect of NGF on CT Distribution between Cytosol and Membranes in Mass-cultured Neurons

The neurons were cultured in 24-well dishes in medium containing 10 ng/ml NGF for 6 days. The medium was then changed: for control neurons, medium containing 10 ng/ml NGF was added; for experimental neurons, medium containing 200 ng/ml NGF was added. After 2 h or 24 h, the medium was removed and the cells were washed twice with cold PBS. For each assay, neurons from 20 wells were combined in cold PBS and pelleted by centrifugation at 100,000 x g_{av} (25,000 rpm, Beckman SW28 rotor) for 1 h. The pellets were homogenized in a glass-Teflon homogenizer using 50 up-and-down strokes in buffer A. The unbroken cells were removed by centrifugation at 500 x g for 10 minutes. The homogenate was further centrifuged at 350,000 x g_{av} (99,000 rpm, Beckman TLA-100.2 rotor) for 15 min to separate the cytosol (supernatant) and the membranes (pellet). The pellet was resuspended in buffer R as the total membranes. The enzyme activities in the cytosol and total membranes were then determined as described in the Materials and Methods section. Krystal's method was used for protein assay. The data were the average of the result from two separate cell preparations.

Time, h	Sample	nmol/min/mg protein	
		10 ng NGF	200 ng NGF
2	Cytosol	1.44	1.26
	Membranes	0.70	0.68
24	Cytosol	1.13	1.15
	Membranes	0.55	0.46

Table 6. Effect of NGF and Anti-NGF Antibody on Distribution of CT Enzyme Activity between Cytosol and Membranes of Mass-cultured Neurons

Neurons were cultured in medium containing 200 ng/ml NGF for 9 days. The medium was then changed: for one group, medium containing 200 ng/ml NGF medium was added; for the other group, medium containing no NGF and 24 nM anti-NGF antibody was added. After 6 h, the samples were collected. The membranes and cytosol were prepared as described in Table 5. For each assay neurons from 20 wells were combined. The enzyme activities of CT were determined as described in the Materials and Methods.

Sample	nmol/min/mg protein	
	200 ng NGF	Anti-NGF Antibody
Cytosol	1.28	1.11
Membranes	0.66	0.57

the activity of CT was assayed in cytosol and membranes. The majority of CT activity was present in the cytosol in both groups (Table 6). The ratio of CT activity on membranes to that in the cytosol in neurons grown in the presence of 200 ng/ml NGF was 0.52 (0.66/1.28) and in the cells grown in the presence of anti-NGF antibody was 0.51 (0.57/1.11). These data indicate that NGF did not directly alter CT distribution between membranes and cytosol in this experiment.

DISCUSSION

1. BIOSYNTHESIS OF PHOSPHOLIPIDS OCCURS IN AXONS OF RAT SYMPATHETIC NEURONS

The biogenesis of membrane components required for axonal growth in neurons is widely believed to occur in cell bodies (Alberts et al., 1989). Turnover and growth of axonal membranes is thought to be dependent upon axonal transport from the cell body for delivery of lipids, proteins and other membrane components (Toews and Morell, 1985; Ledeen, 1985). There is evidence that proteins may be transported from cell bodies to axons via the process of anterograde axonal transport in vesicular structures (Ledeen, 1985) and the assumption has been that lipids of the protein-containing vesicles are co-transported with proteins (Toews and Morell, 1985; Ledeen, 1985). The question of whether or not membrane lipids in mammalian axons can be supplied by synthesis in axons *per se* has not previously been addressed in pure mammalian axons free from other contaminating cell types.

The experiments presented in this thesis show for the first time that significant amounts of the principal lipid components of cell membranes, PC, PE and SM, can be synthesized by living intact axons under conditions in which the neurons can grow and be maintained indefinitely. In axons there exists a whole set of the enzymes - choline kinase, cytidylyltransferase and cholinephosphotransferase - for PC biosynthesis by the CDP-choline pathway. PE can also be synthesized in axons from the CDP-ethanolamine, as well as PC derived from this PE, as shown by incorporation of [^{3}H]ethanolamine into these 2 lipids. Similarly, from radioactive tracer experiments the synthesis of

PS and PE labeled from [3-^{3}H]serine, and of PI labeled from *myo*-[^{3}H]inositol occured in axons.

The apparent biosynthesis of these phospholipids in the axons could not be explained by leakage or diffusion of the radiolabeled precursors from the axon compartment into the cell body-containing compartment since there is virtually no flow of culture medium (Campenot, 1982; Campenot and Draker, 1989) or radioactive precursors between the compartments. Nor could radioactivity in these phospholipids in the axons have been produced by anterograde axonal transport of radiolabeled precursors from the cell body compartment into axon compartments, followed by biosynthesis of the lipid within the cell bodies, and subsequent anterograde movement of the phospholipid products into the axons. After [^{3}H]choline or [^{3}H]ethanolamine was added to the axons, the radioactivity in the water-soluble precursors of the phospholipids, and in the phospholipids themselves, was at all times much higher in the axons than in the cell body-containing compartment. In the cell body-containing compartment, however, there was no detectable radioactivity for the first several hours followed by a gradual increase in that radioactivity. Indeed, the transport of both the phospholipid products and the water-soluble precursors of these phospholipids, between the cell body- and axon-containing compartments was surprisingly slow in both directions.

Cultures raised under conditions similar to those used in the present experiments were assayed for protein using a silver-binding assay for measurement of nanogram quantities of protein (Krystal, 1987). The results showed that the center compartments (cell bodies plus proximal axons) of neurons cultured for 9 days contained 2.5 times as much protein as did the left and right compartments (distal axons) combined. The amounts of proteins

measured were very small and therefore this result must be interpreted with caution because some nerve growth factor, as well as proteins from the serum supplied to center compartments, may have adhered to the cells. Nonetheless, the majority of the neuronal material apparently resided in the center compartment. Consequently, even if the incorporation of [^{3}H]choline into PC and SM, or of [^{3}H]ethanolamine into PE and PC, in the present experiments were considered on the basis of radioactivity per mg cell protein, the data show that only a minor fraction of the phospholipids produced in one compartment was transported into the other compartment. Small amounts of those lipids were apparently transported in both directions. After 16 h of incubation with the radiolabeled precursor the [^{3}H]phospholipids had not equilibrated among the center and side compartments.

Attempts to label phospholipids of the neurons with [^{3}H]glycerol were unsuccessful, apparently because there was minimal uptake of this precursor by the cells.

2. PROTEINS CAN ONLY BE SYNTHESIZED IN CELL BODIES OF PURE SYMPATHETIC NEURONS

It is generally agreed that proteins can only be synthesized in cell bodies, not in axons, and some of these proteins can be transported to axons by anterograde axonal transport (Alberts et al., 1989). Previously, there was a report showing that proteins could be synthesized in a myelinated giant Mauthner axon (Alvarez et al., 1983). The problem in that study was that the myelinated squid axonal fiber was not a pure neuron, but was contaminated with glial cells. The glial cells may have been the source of the labeled axoplasmic protein material since glia-neuron protein transfer has been

demonstrated in myelinated squid giant axons (Gainer, et al., 1977). This means that the incorporation of tracers into macromolecules may occur in glial cells followed by transfer to the axoplasm. By using pure compartmented sympathetic neurons, our study demonstrates that proteins can only be synthesized in cell bodies from [^{35}S]methionine (Fig. 17).

From the present investigation, a revised picture of the mechanism of axonal growth and regeneration has emerged. In the cultured neurons used the axons were presumably elongating at a rate of about 1 mm/day (Campenot and Draker, 1989), which requires a massive input of newly-made phospholipids and proteins for membrane growth. The proteins were apparently supplied by the cell bodies as confirmed in the present study. In contrast, however, phospholipids were synthesized in both the cell body- and axon-containing compartments. The results presented here raise the possibility that membrane phospholipids required for axonal growth and regeneration may be supplied, at least in part, by lipids synthesized *in situ* in the axons. Therefore, an understanding of the regulation of the axonal production of lipids is of prime importance in our knowledge of how nerve fibers grow and regenerate.

3 THE FORMATION OF PC FROM PE METHYLATION IS SIGNIFICANT IN PURE SYMPATHETIC NEURONS

In most mammalian tissues, PC is mainly synthesized via the CDP-choline pathway (Sundler and Åkesson, 1975) whereas PC made from PE *N*-methylation is only quantitively significant in liver where about 20-30% PE can be methylated to PC (Vance, D.E., 1989a; Vance, D.E., 1989b). In other tissues this pathway is only a minor route for PC biosyhthesis (Ridgway, 1989). For

example, it has been reported that in rat brain tissue only a negligible amount of PE was methylated to PC (Ansell and Spanner, 1967; Chojnacki et al., 1964).

Using [1-^{3}H]ethanolamine, this thesis shows that PC is actively derived from PE methylation in pure sympathetic neurons. Fig. 9 shows that after 16 h the ratio of radioactivity from [^{3}H]ethanolamine in PC/PE was 12.2% in cell bodies and 5.9% in axons. The reason for the high ratio of conversion of PE to PC in the compartmented cultured rat sympathetic neurons is not clear, especially in light of the study with rat brain (Dawson, 1985). However, rat brain tissue is not purely neuronal cells but also contains many other cell types (Alberts et al., 1989).

4. PC AND PE ARE THE DOMINANT PHOSPHOLIPIDS SYNTHESIZED IN PURE SYMPATHETIC NEURONS

It was very interesting to look at what kinds of phospholipids were predominantly synthesized in axons. PI, but not PC, had previously been reported to be dominantly synthesized in myelinated squid giant axons when tritiated choline and *myo*-inositol were presented to extruded axoplasm, or injected into the axoplasm of intact giant axons (Gould et al., 1983). Like the squid axon, the myelinated axons of mice were also shown to incorporate *myo*-[^{3}H]inositol into PI (Gould, 1976), and [^{32}P]phosphate (Gould et al., 1978) into phospholipids, far more actively than they did for [^{3}H]choline (Gould and Dawson, 1976), glycerol, or ethanolamine (Gould et al., 1983a). The problem in those experiments, however, was that impure neuron models were used. The myelinated squid giant axons or the myelinated axons of mice contained a glial sheath which is composed of non-neuronal cell types (Gould et al., 1983a). Glial cells can synthesize proteins (Gainer et al., 1977) and lipids (Gould et al., 1987)

and protein and lipids can transfer between the axoplasm and the sheath (Ledeen, 1985). It is therefore difficult to interpret these results.

The results in this thesis demonstrate that the major phospholipids synthesized in both cell bodies and axons of pure sympathetic neurons are PC (from [^{3}H]choline, Fig. 7) and PE (from [1-^{3}H]ethanolamine, Fig. 9). Only small amounts of PS (from [^{3}H]serine in Fig. 12) and PI (from *myo*-[^{3}H]inositol in Fig. 14) were synthesized.

5. NO SYNTHESIS OF CHOLESTEROL OR FATTY ACIDS COULD BE DETECTED IN AXONS OF PURE SYMPATHETIC NEURONS

Cholesterol is ubiquitiously distributed in all eukaryotic membranes and is believed to be indispensable for membrane structure and function (Yeagle, 1985). In addition to being a major source of fuel, fatty acid is also an important component of the phospholipid bilayer (Zubay, 1988). Both cholesterol and fatty acids can be synthesized from glucose and acetate (Zubay, 1988).

Cholesterol biosynthesis in the peripheral nerve (rat sciatic nerve) has been reported (Yao, 1988). The result suggests that *de novo* biosynthesis of cholesterol is one of the major sources of endoneurial cholesterol that forms and maintains peripheral nerve myelin (Yao, 1988). This study, however, did not confirm whether the cholesterol was synthesized by axons or by the glial sheath. *De novo* synthesis of saturated and monounsaturated fatty acids from ^{14}C-labeled glucose and acetate was also claimed to occur in the squid giant nerve fiber (Tanaka et al., 1987). Again these studies failed to show clearly whether or not these lipids were synthesized by axons themselves. It was

possible that the real contributors for the biosynthesis of these neutral lipids was the glial sheaths which are composed of glial cells, axolemma and residual cortical axoplasm (Gould et al., 1983a) since lipid transfer between axoplasm and the sheath has been well documented (Ledeen, 1985). This idea is supported by the results presented in this thesis (Fig. 15). By using compartmented cultures of pure sympathetic neurons from rats, the studies presented in this thesis could not detect any synthesis of cholesterol, or fatty acids from [^{14}C]acetate in axons, even though these lipids were synthesized in the cell bodies. The [^{14}C]acetate was able to enter the axons, therefore a lack of uptake of the radiolabel was not the reason for the apparent lack of synthesis in the axons.

6. CT IS THE RATE-LIMITING ENZYME OF PC BIOSYNTHESIS IN RAT SYMPATHETIC NEURONS

CT is the rate-limiting enzyme of PC biosynthesis in the CDP-choline pathway, as has already been established in liver and other cells (Vance, D.E., 1989a; Vance, D.E., 1989b), but was not previously established in neurons (Dawson, 1985). The results of a pulse-chase study in Fig. 18 show that the reaction catalyzed by CT was the slowest step in the biosynthetic sequence because radioactivity in CDP-choline was very low compared to that in phosphocholine. This confirms that the "bottleneck" in the PC biosynthetic pathway in neurons is at the stage of production of CDP-choline from phosphocholine. Therefore, CT is the rate-limiting enzyme of PC biosynthesis in the intact neurons, also in cell bodies and axons of sympathetic neurons.

7. NO DIRECT EFFECT OF NGF ON CT TRANSLOCATION IN NEURONS

As mentioned in the introduction, NGF is an indispensable neurotrophic factor that affects the development and maintenance of sympathetic, sensory, and specific populations of the neurons in central nervous system (Martinez et al., 1985; Levi-Montalcini, 1987; Barde, 1989; Cattaneo and Mckay, 1990; Ruit et al., 1990). One effect of NGF might be an effect on PC biosynthesis since an important component of the process of axonal growth is production of new membrane material, especially PC.

The preliminary results in Fig. 19 *panel C* suggest that PC biosynthesis may have been stimulated by NGF. The incorporation of [^{3}H[choline into PC was significantly lower in the cells lacking NGF than in the cells grown in the presence of NGF after 12 h and 24 h. Radioactivity was also measured in the water-soluble precursors of PC, choline (*panel A*) and phosphocholine + CDP-choline (*panel B*). The results in Fig. 19, *panel B* suggest that PC biosynthesis may have been increased because there was increase in the radioactivity in phosphocholine + CDP-choline.

There are alternative several possibilities that have caused the apparent increase of PC biosynthesis by NGF. The difference between the label in phosphocholine + CDP-choline in two groups at 0 h in *panel B* might have been caused by the increased influx of labeled choline as seen in Fig. 19, *panel A*.

The growth of neurites from sympathetic neurons is controlled by NGF in the local environment of the neurites (Campenot, 1977). The enzyme CT is the rate-limiting enzyme of PC biosynthesis. Therefore, a mechanism by which NGF promoted PC biosynthesis might have been by stimulating CT translocation from cytosol to membranes. CT exists both on the membranes and in the cytosol in mammalian tissues such as liver (Sleight and Kent, 1980;

Vance, D.E., 1989a) and neurons (Porcellati and Arienti, 1970). The activity of this enzyme in liver is regulated by the reversible translocation of the CT protein between the cytosol (inactive form) and membranes (active form) (Vance, D.E., 1989a; Vance, D.E., 1989b). Thus, the rate of PC biosynthesis can be controlled by factors which either increase or decrease the activity of CT by altering the distribution of the enzyme between membranes and cytosol.

To investigate this possibility, the activity of CT at a high NGF concentration (200 ng/ml) was compared to that at a low NGF concentration (10 ng/ml) in both short term (2 h) and longer term (24 h) experiments. Data presented in Tables 5, however, show that even the high NGF concentration (200 ng/ml) did not increase the translocation of CT from the cytosol to membranes.

The effect of NGF on CT translocation was further investigated by comparing the effect of a high NGF concentration (200 ng/ml) versus no NGF. The latter condition was produced by the addition of medium containing no NGF but with antibody against NGF. The results in Table 6 indicate that in the presence of 200 ng/ml NGF the distribution of CT between cytosol and membranes was no different from the situation in which there was no NGF present. After 6 h, the ratio of CT activity on membranes to that in cytosol in cells treated with 200 ng/ml NGF was 0.52 (0.66/1.28) and in cells treated with anti-NGF antibody was 0.51 (0.57/1.11). All these results suggest that NGF had no direct effect on CT translocation.

It has been demonstrated that NGF affects the gene expression of some enzymes in both sensory and sympathetic neurons (Kessler and Black, 1980; Otten et al., 1980; Thoenen et al., 1971; Mathew and Miller, 1990; Miller et al., 1991), although we still do not know the relation between the effect of NGF on

gene expression and the effect of NGF on local neutite growth control. Administration of NGF to neonates increases the activity of enzymes involved in catecholamine biosynthesis (Thoenen et al., 1971) and enhances the expression of the low affinity NGF receptor, Tα1 α-tubulin, and tyrosine hydroxylase mRNA (Ma et al., 1992). Hence, one hypothesis is that NGF might increase CT gene expression in neurons after treatment with NGF for a longer time (over 12 h) and the increase of PC biosynthesis might be caused by the increase of CT protein. There is no evidence to support this hypothesis at the moment. The mechanism of the effect of NGF on PC biosynthesis is still not clear. Therefore, this needs to be further investigated.

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